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A LIGHT AND ELECTRON MICROSCOPICAL STUDY OF THE MECHANISMS OF  
PATHOGENICITY OF TRICHOMONAS VAGINALIS IN  
EPITHELIAL CELL CULTURES

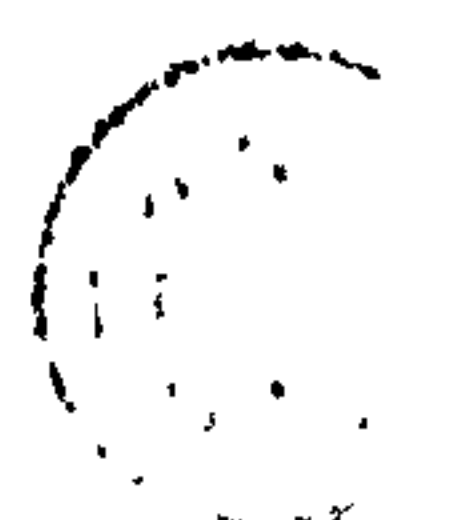
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A thesis submitted for the degree of Doctor of Philosophy in the  
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### ABSTRACT

J. P. HEATH: A light and electron microscopical study of the mechanisms of pathogenicity of Trichomonas vaginalis in epithelial cell cultures.

T. vaginalis is a urogenital protozoan parasite of man, causing the disease known as trichomoniasis. In males the disease is often symptomless; in females acute infections are often associated with inflammation of the cervical and vaginal walls, superficial erosions of the vaginal epithelium and a heavy, purulent vaginal discharge.

In this study I have used an in vitro model of trichomoniasis in order to elucidate some of the mechanisms of pathogenicity of the parasite. The behaviour and cytopathogenicity of T. vaginalis in epithelial cell cultures was examined using phase contrast and interference reflection light microscopy, and scanning and transmission electron microscopy.

The trichomonads attack the epithelial cell monolayers causing pathological changes within the cells which lead to the detachment and lysis of the cells. The lysed cell debris is phagocytosed by the trichomonads. Two aspects of the behaviour of T. vaginalis are of prime importance in the pathogenic processes that lead to the death of the cell cultures:

1) T. vaginalis readily adheres to the exposed surfaces of the epithelial cells and to the glass substratum on which the cells are grown. The adhesions are of the intermediate junction type, characterised by a gap of 10 - 20 nm. Damage to the monolayer of epithelial cells is restricted to those cells with adherent trichomonads.

2) When T. vaginalis adheres to a solid substratum it loses its characteristic spherical shape which is assumed in suspension and it develops pseudopodia which it uses to locomote in an amoeboid manner. The amoeboid trichomonads are capable of migrating between and under the monolayer mechanically breaking the adhesions of the cells to each other and to the substratum. The pseudopodia, and regions of active phagocytosis, of T. vaginalis contain actin-like microfilaments.

Mechanisms that may be involved in the adhesiveness and amoeboid movements of T. vaginalis are discussed, and the possible relevance of these phenomena and of chemical factors to the cytopathogenicity of T. vaginalis in cell cultures and in humans is considered.

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## 1. INTRODUCTION

### 1.1 History of Trichomonas vaginalis and trichomoniasis

In 1836 a French physician, Alfred François Donne, discovered a "microscopic animal" in the vaginal discharge of a woman with severe vaginitis. The animal was the size of a leucocyte but possessed several motile appendages. Donne (1836) named it Tricomonas-vaginale but the name was later amended to Trichomonas vaginalis by Ehrenberg (1838) to follow taxonomic convention. The full scientific name of the urogenital protozoan of man is thus Trichomonas vaginalis Donne, 1836, emend. Ehrenberg, 1838 (Honigberg, 1963; 1978b). Despite Donne's discovery and the subsequent isolations of the parasite from the genital tracts of both women (Scanzoni and Kolliker, 1855) and men (Marchand, 1894) it was not until Hühne (1916) associated the presence of T. vaginalis in the urogenital tracts of humans with specific clinical symptoms that the disease of trichomoniasis was properly recognised. Furthermore, trichomoniasis was still not accepted as a venereal disease until Catterall and Nicol (1960) showed that the primary means of transmission of T. vaginalis between humans was sexual intercourse.

T. vaginalis and trichomoniasis have a world-wide distribution (Honigberg, 1978b). T. vaginalis is primarily a parasite of the human urogenital tract. There is no known non-human animal reservoir of the parasite; however experimental infections may be established in some animals.

### 1.2 Taxonomy of the order Trichomonadida

Trichomonas vaginalis is a member of the phylum Protozoa, sub-phylum Sarcomastigophora, super-class Mastigophora, class Zoomastigophorea, order Trichomonadida (Honigberg et al., 1964a). A detailed discussion of the characteristics and evolutionary relationships of the members of the order Trichomonadida is given by Honigberg (1963) and Brugerolle (1976);

an outline classification is given below.

ORDER TRICHOMONADIDA; General characters:-

Generally 4 to 6 flagella, one of which is recurrent, per mastigont system; undulating membrane, if present, associated with recurrent flagellum; axostyle and parabasal body (= Golgi body) in each mastigont; division spindle extranuclear; sexuality unknown; true cysts unknown; nearly all parasitic or symbiotic.

Four families, each with several sub-families have been described (Brugerolle, 1976). Listed below are the four families, their sub-families and brief details of representative genera and species. Fig. 1a shows simple line drawings of seven species of trichomonads including T. vaginalis; the organisms are drawn to the same scale but it must be noted that there is commonly a wide variation in size, and shape, within each of the species.

A. Family MONOCERCOMONADIDAE

Sub-family MONOCERCOMONADINAE

Monocercomonas (large intestine of reptiles), Hexamastix, Tricercomitus

Sub-family PROTRICHOMONADINAE

Histomonas (H. meleagridis, caecum of turkeys and other galliform birds),

Parahistomonas (P. wenrichi)

Sub-family DIENTAMOEBINAE

Dientamoeba (D. fragilis, large intestine of man)

Sub-family CHILOMITINAE

Chilomitus

Sub-family HYPOTRICHOMONADINAE

Hypotrichomonas, Pseudotrichomonas

B. Family TRICHOMONADIDAE

Sub-family TRICHOMONADINAE

Trichomonas (T. vaginalis, urogenital tract of man; T. gallinae, mouth pharynx, oesophagus, crop of pigeons, turkeys and a wide variety of birds; T. tenax, oral cavity of man)



Tetratrichomonas (Tet. gallinarum, caecum of turkeys, chickens and many other gallinaceous birds)

Pentatrichomonas (P. hominis, large intestine of primates, including man, cats, dogs and rodents)

Trichomitus

Sub-family TRITRICHOMONADINAE .

Tritrichomonas (Tri. foetus, urogenital tract of cattle; Tri. suis, nasal cavity, stomach and intestine of swine; Tri. muris, large intestine of rodents)

Sub-family TRICHOMITOPSIINAE

Trichomitopsis, Pseudotrypanosoma

Sub-family PENTATRICHOMONOIDINAE

Pentatrichomonoides

C. Family DEVESCOVINIDAE

Devescovinia

D. Family CALONYMPHIDAE

Calonympha

### 1.3 Gross morphology of T. vaginalis

When swimming freely in a liquid medium T. vaginalis is normally spherical or ellipsoidal in shape. The size of the organism can vary greatly; measurements made on fixed and stained material generally give smaller values from those made on living organisms from clinical material or cultures (Honigberg, 1978b). The average size of T. vaginalis is 10  $\mu\text{m}$  long by 7  $\mu\text{m}$  wide (Honigberg and King, 1964) but since the body of the organism is very plastic the range of sizes within any population may lie between 4 to 30  $\mu\text{m}$  in length and 2.5 to 15  $\mu\text{m}$  in width.

Figs. 1a and 1b show the gross morphological features of T. vaginalis; the fine structure is discussed later.

#### 1.4 Motility of T. vaginalis

In suspension in a liquid medium T. vaginalis swims by beating the four anterior flagella and undulating membrane but the organism can also move in an amoeboid manner, possibly without the involvement of the flagella, when adhering to a solid substratum (Christian, Miller, Ludovici and Riley, 1963). Until now there has been no detailed study of the amoeboid movements of T. vaginalis.

#### 1.5 Nutrition of T. vaginalis

In natural or experimental infections T. vaginalis feeds on the bacteria, cell debris and tissue fluids present at the site of the infection. Solid material is phagocytosed and nutrients in solution are absorbed by pinocytosis and presumably, although there have been no experimental studies on this aspect, by active or passive transport through the plasma membrane. Electron microscopical studies on human vaginal discharges have shown that trichomonads phagocytose bacteria and epithelial cell debris (Tamayo, Montiel and Garcia, 1972; Nielsen and Nielsen, 1975; Ovcinnikov, Delektorskij, Turanova and Yashkova, 1975).

#### 1.6 Reproduction of T. vaginalis

T. vaginalis divides by longitudinal binary fission. At present the exact details of the division process have not been elucidated but it appears that the first signs of division are the replication of the flagella and their kinetosomes (Hawes, 1947). Brugerolle (1975) has published what is probably the most extensive account to date of the division process in T. vaginalis and other trichomonads. As the two new sets of kinetosomes and their flagella move apart to opposite sides of the body a band of microtubules forms between them; some of the microtubules insert into the nuclear membrane, which remains complete during division. The chromosomes divide and the daughter chromosomes migrate to opposite sides of the nucleus which then divides into two nuclei. It seems likely that the



microtubules are responsible in some way for both the separation of the kinetosomes and the daughter nuclei but clearly further work is necessary before the mitotic mechanisms of T. vaginalis are fully understood. The replication of the other cytoplasmic organelles is followed by cytokinesis and the formation of two separate daughter trichomonads. Brugerolle (1975) has used the term "cryptopleuromitosis" to describe the division process of T. vaginalis.

Multiple fission has never been observed in T. vaginalis but multinucleate forms are commonly seen in cultures (Wirtschafter, 1954; John and Squires, 1978; this study). Such forms may be caused by a random failure of cytokinesis or, as Wirtschafter (1954) suggested, may be due to high oxygen tensions in the culture medium.

The mean generation time of T. vaginalis during the logarithmic phase of growth in culture is generally between 5 and 16 h (Honigberg, 1961; Farris and Honigberg, 1970; Heath, 1972; Perju, Grigoriu, Antonescu and Iercan, 1974).

There is no evidence for a cystic stage in the life-cycle of T. vaginalis.

## 1.7 Fine structure of T. vaginalis

### 1.7.1 Introduction

The first transmission electron microscopical (TEM) studies on the fine structure of T. vaginalis were published twenty years ago by Akashi, Hashimoto, Komori, Mori, Kawasaki, and Tomita (1959), and Inoki, Nakanishi and Nakabayashi (1959). Subsequently Inoki et al. (1960), Ludvik, Stoklosowa and Weglarska (1961), Smith and Stewart (1966) and Yeh Ying, Huang Mei-Yu and Wei-Neng (1966) published similar studies. However all these early reports were incomplete and hampered by poor preservation of fine structure. Significantly, however, these authors noted the absence of mitochondria, a characteristic which was correlated with the anaerobic growth of the protozoon. In 1966, Nielsen, Ludvik and Nielsen produced the

first and as yet the only fully comprehensive description of axenically-cultured T. vaginalis which has served as the main reference work on the fine structure of T. vaginalis. Later reports dealt with the fine structure of T. vaginalis in human vaginal secretions (Tamayo et al., 1972; Nielsen, 1975), and in the tissues of mice (Brugerolle, Gobert and Savel, 1974) and of humans (Nielsen and Nielsen, 1975). The structure of T. vaginalis was redescribed by Ovcinnikov, Delectorskij and Kosmacheva (1974) and Ovcinnikov et al. (1975) in two long and comprehensively illustrated papers but their accounts contained some errors which were pointed out by Heath (1974) and Honigberg (1978b). (Heath, 1974, is at end of thesis).

In contrast to the numerous TEM studies, there have been surprisingly few scanning electron microscopical (SEM) studies on T. vaginalis. Ovcinnikov et al. (1975), Kazanowska, Jodczyk, Kuczynska and Karpowicz (1977), Kurnatowska and Hajdukiewicz (1977), John and Squires (1978) and Warton and Honigberg (1979) have published scanning electron micrographs of T. vaginalis but none of these papers was particularly detailed or comprehensive.

The following description of the fine structure of T. vaginalis is based on the TEM and SEM studies published to date and, although it does not include the results of this study, is illustrated by my own electron micrographs. Fig. 1b shows the spatial relationship of the main organelles of T. vaginalis.

#### 1.7.2 Plasma membrane

In glutaraldehyde- and osmium-fixed organisms, the plasma membrane has the typical trilaminar structure (Fig. 38), and is about 7 nm thick. Pinocytosis occurs all over the surface of the body; pinocytotic pits and cytoplasmic pinocytotic vesicles generally possess an internal fibrillar surface coat 20 to 40 nm thick (Filadoro, 1970), (Fig. 69), but it is a contentious point as to whether this surface coat is always present on the remainder of the plasma membrane. Nielsen et al.'s (1966) electron micro-



graphs of cultured T. vaginalis show no such coat. However Filadoro (1969) reported that cultured organisms fixed and stained with ruthenium red and lanthanum dyes, without prior washing to remove the serum-containing culture medium, possess a surface coat up to 35 nm thick which stains strongly with the dyes, indicating that the coat contains anionic polysaccharides. The coat was only seen on a small area of the plasma membrane of each organism. Later Filadoro (1970) stated that the coat covered the whole surface of the organism and was continuous with the internal coat of pinocytotic vesicles during their invagination. Ovcinnikov et al. (1975) also observed that T. vaginalis fixed directly in human vaginal discharges and stained with ruthenium red exhibited a continuous 30nm thick surface coat, but the coat was not seen in unstained trichomonads. Other authors have noted that only small parts of the membrane possess a coat which may be filamentous (Nielsen, 1970; Nielsen and Nielsen, 1975) or amorphous (Brugerolle et al., 1974); these observations were made on T. vaginalis taken from both culture and from natural and experimental infections making it unlikely, by analogy with the trypanosomes (Vickerman and Preston, 1976), that the organism only adopts a surface coat within its host. Until there is unequivocal evidence to the contrary, it seems likely that T. vaginalis does not possess a surface coat and that the material sometimes seen on the plasma membrane is adsorbed from the serum or tissue components surrounding the parasite; an opinion which is shared by Brugerolle et al. (1974).

### 1.7.3 Flagella, undulating membrane and accessory structures

T. vaginalis has four anterior flagella each about 10  $\mu$ m long and a fifth recurrent flagellum which passes posteriorly attached to a thin fold of cytoplasm called the undulating membrane. The kinetosomes of the flagella lie in the anterior pole of the cell at the bases of the flagella; those of the four anterior flagella have their long axes parallel and are distributed radially around the fifth kinetosome of the recurrent

flagellum which is tilted at about  $90^\circ$  with respect to the others (Fig. 84). The structure of the flagella and kinetosomes is identical to that of the cilia and flagella of many other protozoa (Sleigh, 1973).

The kinetosomes are closely associated with several fibrous structures, the whole complex being called the mastigont system. Full details of the mastigont systems of T. vaginalis and other species of trichomonads and pertinent references are given by Honigberg (1978a; 1978b). The largest fibrous structure is the costa which is rod-shaped,  $10\text{ }\mu\text{m}$  long by  $1\text{ }\mu\text{m}$  wide, and passes backwards from the kinetosome of the recurrent flagellum running beneath the undulating membrane (Fig. 38); its function may be to support the undulating membrane.

The undulating membrane is a fold of the plasma membrane which arises at the point of emergence of the anterior flagella from the anterior pole of the trichomonad and runs posteriorly for about half the length of the cell (Figs. 14 and 17). It protrudes for a distance of  $0.5 - 1.0\text{ }\mu\text{m}$  from the body of the trichomonad. The distal edge of the undulating membrane forms the marginal lamella which contains electron dense material but the proximal portion encloses little structured cytoplasmic material (Figs. 35 and 37). The recurrent flagellum is attached to the undulating membrane at the proximal edge of the marginal lamella but no specialisation of the apposed membranes nor any material between them has been seen (Fig. 37).

#### 1.7.4 Other cytoplasmic organelles and inclusions

The nucleus is ellipsoidal and located in the anterior half of the cell. The nucleus is about  $4\text{ }\mu\text{m}$  long by  $2\text{ }\mu\text{m}$  wide and is surrounded by a double unit membrane which is continuous with the rough endoplasmic reticulum which forms a corona around the nucleus (Figs. 35 and 36).

The axostyle is made up of a single sheet of parallel microtubules (Fig. 43) which runs from the kinetosomes down the length of the body and projects from the posterior pole for a distance of up to  $10\text{ }\mu\text{m}$  (Figs. 10 and 71). At the anterior pole of T. vaginalis the axostylar sheet is



planar but as it passes the nucleus it becomes rolled up longitudinally like a scroll. The number of microtubules in the axostyle is gradually reduced from about 55 at the anterior pole to less than 10 in the axostylar projection. In cross section the microtubules are linked by lateral cross bridges which may maintain the integrity of the sheet.

The Golgi body, also called the parabasal apparatus or body by light microscopists (Honigberg and King, 1964), lies on the opposite side of the nucleus from the axostyle and between the nucleus and the costa (Fig. 35). The Golgi body consists of a stack of parallel flattened, membrane-bound, cisternae (Fig. 39). According to current theories on the role of the Golgi body in cell physiology (Cook, 1973), this organelle has two physiologically different sides. The "forming face" receives vesicles containing newly synthesised material from the endoplasmic reticulum; after further metabolism the material is released in vesicles into the cytoplasm from the "secretory face" to perform its various functions (Fig. 39).

The cytoplasm of T. vaginalis contains a population of spherical granules each approximately 0.5  $\mu\text{m}$  in diameter (Fig. 35). Light microscopists called these structures the paracostal and paraaxostylar granules from their juxtaposition to those organelles in stained preparations of trichomonads. Following electron microscopical and biochemical studies these granules have been renamed hydrogenosomes since they are the sites of terminal respiration and hydrogen evolution (Lindmark, Muller and Shio, 1975).

Hydrogenosomes have an electron dense but structureless matrix surrounded by a limiting unit membrane and they are scattered throughout the cytoplasm occupying about 6 per cent of the volume of T. vaginalis (Nielsen and Diemer, 1976).

The remaining cytoplasmic inclusions include large vesicles 0.5 to 2.0  $\mu\text{m}$  in diameter some of which contain particulate material and are

phagosomes (Fig. 35), numerous free ribosomes and many glycogen granules which are reserves of carbohydrates. (Figs. 37 and 38).

#### 1.7.5 Pseudopodia

Many authors have noted from both light and electron microscopical studies that T. vaginalis is capable of extending small irregularly-shaped processes - pseudopodia- from its body. Using light microscopy, Honigberg and King (1964) noted that living organisms used pseudopodia to ingest particulate material and to attach themselves to solid objects. Pseudopodia are only infrequently seen in cultured T. vaginalis and so it is only relatively recently, when investigators examined trichomonads in vaginal secretions and in tissue, that their fine structure was revealed. Typically the pseudopodia of T. vaginalis contain few cytoplasmic organelles but instead contain an abundance of 5 nm diameter microfilaments (Figs. 60 and 63) which form a 1 to 2  $\mu$ m thick layer beneath the plasma membrane; Nielsen and Nielsen (1975) called this layer "ectoplasm" from its similarity to the cortical cytoplasm of rhizopod amoebae (see Jeon, 1973).

### 1.8 Physiology and biochemistry of T. vaginalis

#### 1.8.1 Anaerobic metabolism

T. vaginalis is a predominantly anaerobic protozoan. It has no mitochondria, no cytochrome c and so the trichomonad has very low  $O_2$  consumption which is cyanide and azide insensitive (Baernstein, 1963). The tri-carboxylic acid cycle is inoperative in T. vaginalis; of the enzymes in this pathway only malic and succinic dehydrogenases have been found (Baernstein, 1963; Brugerolle and Metenier, 1973). Growth in media exposed to air decreases the activity of glycolytic enzymes; growth under  $N_2$  increases their activity (Arese and Cappuccinelli, 1974). Although it will survive in aerobic conditions, high oxygen tensions are toxic due to the production of  $H_2O_2$ , T. vaginalis having no catalase. Thus most culture media contain a reducing agent such as cysteine or thioglycollate.



### 1.8.2 Carbohydrate metabolism and energy production

T. vaginalis obtains most of its energy by the anaerobic metabolism of carbohydrates, glycolysis being the major metabolic pathway. The chief end products of fermentation are lactic acid, malic acid, acetic acid, and the gases  $\text{CO}_2$  and  $\text{H}_2$ .

The main carbohydrates used by T. vaginalis are glucose and its polymers maltose, starch and glycogen. Read (1957) showed that maltose is a better substrate than glucose yielding 5 times as many organisms in culture and he also observed that glucose polymers with a  $\beta$ -1.4-glucoside linkage were required for optimum growth. All of the enzymes of the glycolytic pathway are found in the non-sedimentable fraction of homogenates of T. vaginalis (Muller and Lindmark, 1974). The pentose phosphate pathway is also used for glucose metabolism providing precursors for nucleic acid synthesis (Arese and Cappuccinelli, 1974). Pyruvate breakdown involves at least two pathways. Firstly, pyruvate is metabolised to lactate by a NAD-linked lactate dehydrogenase, and secondly a phosphoroclastic-type pathway is used in which pyruvate is decarboxylated to Acetyl-CoA and  $\text{CO}_2$  by pyruvate synthase (Edwards, Dye and Carne, 1973). This latter pathway generates electrons (linked to an electron transfer protein, possibly ferredoxin or flavodoxin) which are combined with protons by a hydrogenase located in the hydrogenosomes of T. vaginalis to give molecular  $\text{H}_2$  (Lindmark and Muller, 1974a). The pathway leading to the formation of  $\text{H}_2$  is one of the sites of action of the trichomonacidal drug metronidazole (Lindmark and Muller, 1974b).

Detailed and comprehensive reviews of anaerobic carbohydrate metabolism in T. vaginalis have been presented by Shorb (1964) and Honigberg (1978b).

### 1.8.3 Other metabolic pathways

T. vaginalis is one of the non-chlorophyll containing protozoa capable of fixing  $\text{CO}_2$ .  $^{14}\text{C}$  - labelled  $\text{CO}_2$  is incorporated in to the carboxyl

group of lactic acid (Wellerson, Doscher and Kupferberg, 1960).

De novo synthesis of amino acids takes place in T. vaginalis despite its complex nutritional requirements. When given  $^{14}\text{C}$ -labelled glucose or succinate the radioactivity was subsequently detected in amino acids and proteins (Kunitake, Stitt and Saltman, 1962).

T. vaginalis cannot itself synthesise sterols and other classes of lipids and so must obtain them from its environment, such as from the serum component of culture media (Etinger and Halevy, 1965; Roitman, Heyworth and Gutteridge, 1978).

#### 1.8.4 Hydrolytic enzymes

Biochemical and cytochemical studies on both whole organisms and homogenates of T. vaginalis have demonstrated the presence of at least five hydrolytic enzymes which are able to digest many biomolecules and may be involved in tissue destruction in experimental and natural infections.

Acid phosphatase and  $\beta$ -N-acetylglucosaminidase, which are enzyme markers for lysosomes are predominantly located in a population of lysosomal-like membrane-bound vesicles from 0.1 to 5.0  $\mu\text{m}$  in diameter; the smaller of these bodies are probably derived from the Golgi body, the larger are phagosomes (Takeuchi et al., 1972; Nielsen, 1974; Lindmark, et al., 1975). Sharma and Bourne (1964) demonstrated  $\beta$ -glucuronidase activity in T. vaginalis using a cytochemical technique, however Fishman, Kasdon and Homburger (1950) were unable to detect this enzyme biochemically in homogenates of trichomonads. Hyaluronidase activity is present in homogenates of T. vaginalis and the enzyme is also secreted by living organisms (Boni and Orsi, 1958; Filadoro and Orsi, 1960).

#### 1.9 Laboratory culture of T. vaginalis

Many different media have been devised for the axenic culture of T. vaginalis. For details of recipes of the more common media see Lumsden et al. (1966), Taylor and Baker (1968), Jirovec and Petru (1968) and Hess (1969).



The main energy source required by T. vaginalis in culture is glucose or maltose. Growth factors are supplied by the addition of about 10 per cent serum, generally human, horse or foetal bovine serum, although serum-free defined media have been devised for trichomonads (Lee, Ast, Hutner and Allen, 1964). Since T. vaginalis is an anaerobe, culture media include a reducing agent such as cysteine hydrochloride or sodium thioglycollate, and the medium is placed in tightly stoppered bottles. For the primary isolation of T. vaginalis from humans, when bacteria and fungi may be present, penicillin (1000 U/ml), streptomycin (500 U/ml) and a fungicide are often also included. It has been shown, however, that antibiotics may alter the antigenic character of T. vaginalis (Kott and Adler, 1961). Proteins and amino acids are supplied by the addition of a digest of liver or other tissue.

The optimum pH for the growth of T. vaginalis is between 5.8 and 6.8 (Lumsden et al., 1966; Heath, 1972) and media are buffered accordingly. The optimum temperature for growth is 37°C but growth proceeds more slowly but still adequately at 28°C (Abarbachuk and Voronina, 1969). Lower temperatures generally inhibit multiplication (Jirovec and Petru, 1968).

T. vaginalis may also be grown in semi-solid media (Lowe, 1972) or on solid media made with agar (Ivey, 1961); in the latter situation the trichomonads grow as colonies and use of this characteristic has been made for cloning (Samuels, 1962). T. vaginalis will also grow as colonies on mycoplasma medium (Andrews and Thomas, 1974).

In suitable media trichomonads will multiple to a density of 1 to 2 x 10<sup>6</sup> cells per ml when the cultures enter the stationary phase of growth due to the inhibitory effect of the low pH (about 4) caused by the acidic products of fermentation such as lactic acid.

## 1.10 Epidemiology of human urogenital trichomoniasis

### 1.10.1 Prevalence

Many people have argued that trichomoniasis is the commonest of the

primarily sexually transmitted diseases (Morton, 1975; Honigberg, 1978b). Catterall (1972) estimated that about 1 million women in the UK were infected with T. vaginalis, and Morton (1975) estimated that one woman in every five is infested at some time during her sexually active years. These judgements are not however always sustained by the statistics available in the UK. Table 1 gives data on the prevalence of trichomoniasis in England from 1973 to 1977 and shows that gonorrhoea, for example, is more prevalent than trichomoniasis in both males and females. On the other hand, these statistics are collated from venereal disease clinics only and so do not include the many cases of trichomoniasis treated by general practitioners (Dr. V. J. Heath, personal communication). It is clear however from Table 1 that trichomoniasis and other sexually transmitted diseases are becoming increasingly prevalent. Another significant feature of the data in Table 1 is that trichomoniasis is approximately twelve times more frequently diagnosed in females than males. This disparity is probably because infected males are commonly symptomless and have few parasites making diagnosis more of a problem (Morton, 1975).

Comprehensive reviews of the prevalence of trichomoniasis throughout the world are given by Jirovec and Petru (1968), Morton (1975) and Honigberg (1978b).

#### 1.10.2 Transmission

In the vast majority of cases T. vaginalis is transmitted by sexual intercourse; the rarity of trichomonal infections in babies (Bramley, 1976), pre-pubertal children and virgins (Jirovec and Petru, 1968) only emphasises this fact. Nevertheless, as Honigberg (1978b) points out, in some cases of trichomoniasis a non-venereal route is the only possible way in which the infection could be acquired. Al-Salihi, Curran and Wang (1974) and Postlethwaite (1975) have documented cases of trichomoniasis in neo-natal boys and girls who were delivered of mothers with the disease and were probably infected during parturition. The ability of T. vaginalis to survive in tap water at 21°C for 12 h (Honigberg, 1978b), and on moist



towels at room temperature for 23 h (Burch, Rees and Reardon, 1959) makes it at least theoretically possible that infections may be acquired non-venereally from infected persons by the sharing of baths and towels.

### 1.11 Trichomoniasis in females

#### 1.11.1 Infection sites

The most common and typical site of T. vaginalis is the vagina. In about 15 per cent of women with trichomonal vaginitis T. vaginalis is also found in the urethra and bladder (De Leon, 1971; Honigberg, 1978b). In some instances parasites are also found in Skene's ducts and in Bartholin's glands (Honigberg, 1978b). Trichomonads do not normally ascend above the external os of the cervix unless there is other disease present (Allen and Butler, 1946). Grys (1966) suggested that the mucus-filled endocervical canal presented a physical barrier to entry of T. vaginalis into the uterus from the vagina.

#### 1.11.2 Symptoms

Many infected women are symptom-free and others have only a slight vaginal discharge (Schofield, 1972).

In acute infections, named florid vaginitis (Frost, 1962), there is a copious and foul smelling greenish-yellow vaginal discharge. The vulva, perineum and patches on the inner sides of the thighs are often inflamed (Schofield, 1972) and the patient may complain of an itching and a burning sensation in and around the genitals (De Leon, 1971). Typically the vaginal walls and the cervix are inflamed and oedematous and there are often minute petechial haemorrhages of the vagina and cervical epithelia (Jirovec and Petru, 1968; De Leon, 1971); the inflammation gives rise to a characteristic strawberry-red appearance of the cervix and vagina. The surface of the cervix is also often covered with the discharge which collects in pools in the posterior fornix (De Leon, 1971). When the urethra and bladder are infected dysuria and urinal urgency are common (King and Nicol, 1975) and the urine often contains mucus and leucocytes

(Glebski, 1965). Complications include bartholinitis, skenitis, cystitis (Schofield, 1972) and infected women often complain of dyspareunia (Honigberg, 1978b). Exacerbation of the symptoms may occur during the menses (De Leon, 1971), during pregnancy (Schofield, 1972) and in the puerperium (Nielsen and Nielsen, 1975).

If the disease is not treated the acute or florid phase may be followed by an asymptomatic latent phase (Frost, 1962) in which the parasites are present only in the endocervical canal (De Leon, 1971), causing a chronic endocervicitis.

### 1.11.3 Pathological changes in the cervix and vagina

#### 1.11.3.1 Introduction

We now know a good deal about the pathological changes in the vaginal and cervical tissues that occur in infected women. However the mechanisms by which T. vaginalis causes these changes is less clear. Extensive histological (based on tissue biopsies) and cytological (based on smears) light microscope studies have been performed by, among others, Trussel (1947), Bechtold and Reicher (1952), Koss and Wolinska (1959), Frost (1962), Lotoki (1962) and Frost (1974) and the following sections are based on their findings. The electron microscopical studies of Nielsen and Nielsen (1975) and Garcia-Tamayo et al. (1978), because of their direct relevance to this thesis, are treated in a separate section (1.11.3.5).

In about a third of women with trichomonal vaginitis no histological or cytological abnormalities are found (Koss and Wolinska, 1959).

#### 1.11.3.2 Histology of the normal cervix and vagina

Histologically the cervical and vaginal walls are comprised of three layers, an inner mucous membrane or mucosa, which lines the cervico-vaginal vault, a central muscular layer and a surrounding connective tissue sheath (Novak, Jones and Jones, 1965). The mucous membrane consists of the lamina propria, containing connective tissue and blood vessels, and the superficial epithelial layer. The epithelial layers of the vagina and



ectocervix are made up of a 150 to 200  $\mu\text{m}$  thick layer of non-cornified stratified squamous epithelial cells; the outermost cells contain large amounts of glycogen which is metabolised by the vaginal flora giving rise to the acidic (pH 4 to 5) conditions in the vagina. Finger-like processes, or papillae, containing connective tissue and capillaries project into the stratified epithelial layers from the lamina propria. Unlike the vagina and ectocervix, the endocervix is lined by a single layer of columnar epithelial cells which secrete most of the mucus which lubricates the vaginal walls.

#### 1.11.3.3 Changes in the vaginal and ectocervical mucosae

The most common finding in the vaginal and ectocervical mucosae is a sub-epithelial inflammation with an increase in the vascularity of the papillae. The papillae are often elongated with their inflamed tips separated from the surface by only a few layers of epithelial cells. The clinical appearance of the so-called strawberry vagina and cervix is considered by many authors to be due to the increased vascularity of the papillae and the focal extravasation of blood at their tips (Honigberg, 1978b).

Oedema of the squamous epithelium frequently occurs, causing a partial separation of the epithelial cells which subsequently remain attached to their neighbours by only thin cytoplasmic bridges, giving the epithelium a chicken-wire appearance (De Leon, 1971). The spaces between the cells are infiltrated by leucocytes. The epithelium may be thinner than normal due to an increase in the rate of desquamation of the peripheral layers (Lotoki, 1962); the denuded cells may be enlarged, irregular in shape and occasionally bi-nucleate. Cellular necrosis of the epithelial surface is also common, however necrotic ulceration of the entire epithelial thickness is only rarely seen.

The most frequently encountered cytological changes in the epithelial layers are:

- a) an intracellular vacuolisation which largely occurs around the nucleus

causing a transparent perinuclear halo, and b) enlargement of the nucleus with associated abnormalities of the chromatin, such as margination and pyknosis (Koss and Wolinska, 1959). These changes are more common in the outer layers of the epithelium especially if the surface is covered with purulent vaginal discharge. Only rarely are the basal epithelial cells affected and in such cases they show a slight hyperplasia.

#### 1.11.3.4 Changes in the endocervical mucosa

Abnormalities of the endocervical mucosa are not often seen possibly because the parasites have difficulty in penetrating the mucus-filled endocervical canal (Grys, 1966). Occasionally the columnar epithelium may be replaced by squamous epithelial cells of varying maturity; in other cases there may be a slight inflammation of the columnar epithelium which may contain a few abnormal or hypersecretory cells (Koss and Wolinska, 1959; Frost, 1974).

#### 1.11.3.5 Interaction of *T. vaginalis* and vaginal and cervical epithelia

The early light microscope studies on the histological changes associated with trichomonal vaginitis made little progress in elucidating the mechanism whereby *T. vaginalis* caused the observed changes.

Koss and Wolinska (1959) were unable to find *T. vaginalis* within the epithelium of the cervix of women with trichomonal vaginitis although they did find the parasite trapped within the discharge covering the cervical surface and both they and Bechtold and Reicher (1952) observed groups of trichomonads adhering to desquamated cells. Frost, Honigberg and McLure (1961) observed *T. vaginalis* in a non-phagocytic squamous epithelial cell and suggested that the parasite was capable of invading healthy cells. Nevertheless at that time it remained an open question whether *T. vaginalis* actually adhered to and/or invaded the epithelium and attacked cells or alternatively exerted its injurious effects indirectly by secreting toxic factors.

Some light was shed on this problem following transmission electron



microscopic studies of tissue biopsies (Nielsen and Nielsen, 1975), vaginal scrapes (Garcia-Tamayo et al., 1978) and vaginal secretion (Tamayo et al., 1975) from infected women. A significant finding, common to all these studies, was the high frequency with which amoeboid T. vaginalis were found adhering, often in groups, to squamous epithelial cells. The parasites often lay in very close contact with the cells; in some areas of the contact the apposed plasma membranes of the trichomonads and cells were separated by a gap of 10 to 15 nm, placing these contacts in the class of intermediate-type adhesive junctions (Trinkaus, 1969). At the contacts with the cells the trichomonads' cytoplasm was differentiated and contained a meshwork of microfilaments 2 to 5 nm in diameter within which bundles of microfilaments lay, commonly situated at right angles to the contact. In many instances short microfilament-containing pseudopodia interdigitated with processes from the epithelial cells; these pseudopodia appeared to be nipping off parts of the cell cytoplasm and indeed epithelial cell constituents were seen within phagosomes in the cytoplasm of the trichomonads indicating that phagocytosis of the cells was taking place. Nielsen and Nielsen's (1975) study of cervical biopsies showed amoeboid T. vaginalis often adhered to the under surface of partly desquamated epithelial cells which showed necrotic changes. They concluded that the parasites adhered only to necrotic cells but they did not consider the possibility that the necrotic changes may have occurred after the parasites had adhered to the cells. Nielsen and Nielsen (1975) did not find T. vaginalis within the intact non-desquamating epithelium but they did find minor clusters of the organisms lying in shallow depressions of the epithelial surface which suggests that the organisms may be capable of eroding the superficial epithelial cells. These authors noted a slight hyperplasia of the cervical epithelium accompanied by an epithelial inflammation with an invasion of neutrophils which were located in lacunae between the epithelial cells. Although they did not detail the cytological changes in the epithelium

they noted that severe inflammation was accompanied by hyperchromasia and nuclear enlargement of the cells.

The important finding of these EM studies was that T. vaginalis is capable of adhering to the superficial cells of the vaginal and cervical epithelia and so exerting its injurious effects at close range however, as Nielsen and Nielsen (1975) pointed out the widespread inflammatory response in infected patients and the occurrence of cytological changes in the less superficial layers, away from the direct influence of the parasites, indicates the additional involvement of diffusable parasite-derived toxic factors in the pathogenicity of T. vaginalis.

## 1.12 Trichomoniasis in males

### 1.12.1 Infection sites

The most commonly infected sites in males are the urethra and the prostate gland (Jiroves and Petru, 1968; Schofield, 1972; Morton, 1975; Honigberg, 1978b). In uncircumsised men T. vaginalis may be found in the subpreputial sac (Schofield, 1972). There is little clear evidence that the parasite invades any other urogenital organs. Although trichomonal infections in men may be associated with an epididymitis (Amar, 1967; Fisher and Morton, 1969), haemospermia (Walton, 1969), and the presence of parasites in the semen (Whittington, 1951; Bernfeld, 1972), these signs and symptoms do not necessarily imply that T. vaginalis is capable of infecting the seminal vesicles, epididymes and testes since firstly, as Amar (1967) noted, inflammation of the prostate could cause a reflux of urine into the vas deferens and epididymes thus causing a secondary inflammation of these organs, and secondly parasites could contaminate the semen as it passes down the urethra during ejaculation.

### 1.12.2 Symptoms

Men are frequently symptomless carriers of T. vaginalis (Schofield, 1972). In acute cases there is a muco-purulent urethral discharge, the early morning urine is cloudy and in uncircumsised men there may be a



sub-preputial discharge. Itching of the penis, prostatitis, discomfort during urination and painful erections are also common findings (Schofield, 1972; Honigberg, 1978b). Other, less common, symptoms include epididymitis, haemospermia, inguinal adenitis and littritis (Schofield, 1972; Honigberg, 1978b). Persistent infections in men are commonly associated with some degree of urethral stricture (Weston and Nicol, 1963) which, by restricting the flow of urine, may reduce the rate at which the parasites are expelled from the <sup>urethra</sup> ~~urethra~~. Morton (1975) regards urination as important in making trichomoniasis a short-lived affection in many men.

### 1.12.3 Pathological changes

Some indication of the paucity of our knowledge about what, if any, pathological changes occur in the organs of infected males is given by the fact that no mention of this subject is made in the reviews of trichomoniasis by Jirovec and Petru (1968), De Leon (1971) and Honigberg (1978b). However some studies have been made on the role of T. vaginalis in infertility in men. Kolesow (1950) observed that T. vaginalis phagocytosed spermatozoa in trichomonad-infected semen and more recently Tuttle, Holbrook and Derrick (1977) found that a striking decrease in spermatozoal motility occurred within 6 h at 37°C when 0.2 ml semen was mixed with an equal volume of Diamond's broth containing  $10^6$  or  $10^7$  T. vaginalis per ml.; similar experiments with  $10^4$  and  $10^5$  organisms per ml caused only a minor retardation in spermatozoal motility. These studies showed that T. vaginalis is potentially pathogenic to spermatozoa and indeed some infertile men have been found harbouring the parasite in their semen (Bernfeld, 1972) but nevertheless no-one has yet shown that eradication of trichomoniasis in sub-fertile men enhances fertility, a necessary step if we are to implicate T. vaginalis as a cause of male infertility.

## 1.13 Diagnosis of trichomoniasis

### 1.13.1 Collection of material from patients

In women the most fruitful source of trichomonads is the pool of

secretion found in the posterior fornix of the vagina. This material may be collected with a pipette, a charcoal impregnated cotton wool swab or soaked up into a polyester sponge (Robertson, Lumsden, Fraser, Hosie and Moore, 1969; Oates, Selwyn and Breach, 1971). In men the best sources are the urethral discharge, urethral scrapings, a centrifuged deposit of early morning urine, semen and prostatic fluid.

Once the material has been collected from the patient there is a variety of techniques available to the clinician and parasitologist for the demonstration of T. vaginalis. There has been considerable disagreement among workers as to which diagnostic method is the most sensitive (see reviews of Honigberg, 1978b), and Jirovec and Petru, (1968), but preferably at least two, more if possible, different methods should be employed to ensure a correct diagnosis; the two methods most commonly used are wet film microscopy and culture. Robertson et al. (1969) showed that the combined use of wet film and culture methods gives the greatest number of correct diagnoses.

#### 1.13.2 Wet film microscopy

A sample of the material collected from the patient is examined directly, or after mixing with a drop of normal saline, on a slide by phase contrast microscopy. Trichomonads retain their motility for several hours at room temperature and can be distinguished from leucocytes and epithelial cells by their undulating membranes and beating flagella. An improved sensitivity of wet film examination was obtained by Robertson et al. (1969) who showed that centrifugation of vaginal secretions, thereby concentrating the density of T. vaginalis in the material used for wet film examination and for inoculating cultures, increased the number of correct diagnoses in females.

#### 1.13.3 Culture

A specimen from the patient is placed in a bottle of Trichomonas culture medium. Cultures are incubated at 37°C and examined daily for the



presence of T. vaginalis (Lumsden et al., 1966). Inoculated cultures should be incubated for at least seven days or more since many primary isolates of T. vaginalis, especially if they are from pathogenic strains,<sup>1</sup> grow only slowly in culture (Whittington 1957; Kulda et al., 1970).

The sensitivity of cultural methods for the diagnosis of trichomoniasis may be determined by the composition of the culture media (Hess, 1969; Lowe, 1972; Rayner, 1968) and the number of trichomonads in the cultured sample (Rayner, 1968).

#### 1.13.4 Other diagnostic methods

Other methods available for the diagnosis of trichomoniasis include both protozoological methods, such as the microscopic examination of fixed and stained smears of material collected from patients, and serological or immunodiagnostic techniques (see Honigberg, 1978b for review). The latter have been employed in two ways. Firstly by using fluorescently labelled antibodies to T. vaginalis to detect the parasite in clinical material (Hayes and Kotcher, 1960), and secondly, and potentially more importantly, by diagnosing the disease by demonstrating an immune response to T. vaginalis in the patient (Jirovec and Petru, 1968; Ackers, Lumsden, Catterall, and Coyle, 1975; Ackers, Catterall, Lumsden and McMillan, 1978). Although none of these methods has so far proved to be superior to wet film examination and culture, immunodiagnostic methods may yet be of value in asymptomatic infections or in cases where parasite numbers are low; however, serological methods have the drawback that they may detect both current and earlier, eliminated infections.

#### 1.14 Treatment of trichomoniasis

The most widely used and successful drug for the treatment of trichomoniasis is metronidazole (Flagyl; May and Baker Ltd.). Prior to its dis-

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1. Where the author of a work cited in this thesis has used the term "strain" to designate a particular population of T. vaginalis, the designation is retained.

covery the treatment of the disease was always a problem and cure rates were low. For reviews of the history of the treatment of trichomoniasis see Trussel (1947), Jirovec and Petru (1968), Michaels (1968) and Forgan (1972).

Metronidazole, I-(2-hydroxyethyl)-2methyl-5nitro-imidazole, was first synthesised and shown to be trichomonacidal by Cosar and Jolou (1959). Metronidazole has a very low toxicity; the  $LD_{50}$  for mice with a single oral dose is 4.3 g/kg. The drug has a wide spectrum of activity and is also used for the treatment of anaerobic bacterial infections, amoebiasis, giardiasis, balantidiasis, Vincent's stomatitis, Crohn's disease and for some nematode infestations (Ings, McFadzean and Ormerod, 1974; Tanowitz, Wittner, Rosenbaum and Kress, 1975; Roe, 1977). Its action is limited however to infections with anaerobic or facultatively aerobic organisms (Coombs, 1973).

The standard regime for the treatment of trichomoniasis in both men and women is 200 mg metronidazole orally, 3 times daily for 7 days (Catterall, 1972). Cure rates of between 90 and 100 per cent are common with the standard regime (Feo and Fetter, 1961; Morton, 1975). A single oral dose of 2 g metronidazole gives cure rates of between 82 and 100 per cent (Morton, 1975).

We now know a good deal about the mechanisms of action of metronidazole. In T. vaginalis, metronidazole competes with pyruvate synthase and hydrogenase for low redox potential electrons thus inhibiting the formation of molecular hydrogen in the hydrogenosomes (Lindmark and Muller, 1974b). The evolution of  $H_2$  is inhibited before  $CO_2$  evolution suggesting that interference with the terminal electron acceptors is the primary action of the drug (Edwards and Mathison, 1970); both the nitro- group and the imidazole ring of metronidazole give the drug the properties of an electron sink. This may not be the only mechanism of action of metronidazole in T. vaginalis since the reduced metronidazole, possibly as a hydroxylamine, goes on to react with DNA to inhibit the action of DNA and RNA polymerases



(Ings et al., 1974). Nucleic acid synthesis is inhibited within 30 min of exposure of T. vaginalis to solutions containing 5 µg per ml metronidazole and death of the parasite occurs about 5 h later (Ings et al., 1974). Nielsen (1976) noted that cell division was inhibited within 1 h by 1 to 4 µg per ml metronidazole. It is likely that both of the mechanisms described above contribute to the death of T. vaginalis in the presence of metronidazole.

Other drugs used to treat trichomoniasis are, like metronidazole, all derivatives of nitroimidazole, and include nitrimidazine (De Carneri et al., 1969), tinidazole (Wallin and Forsgren, 1974), ornidazole (Skold, Gnarpe and Hillstrom, 1977) and carnidazole (Notowicz, Stolz and De Konig, 1977). Clinical trials have shown that these drugs are generally as efficacious as metronidazole.

### 1.15 Pathogenicity of T. vaginalis in experimentally infected animals

#### 1.15.1 Introduction

Experimental models of trichomonal infections using animals have been studied for many years as a method of elucidating and comparing the pathogenicity of strains of T. vaginalis and for the testing of therapeutic agents. The most extensively used animals have been mice and guinea pigs. The results of T. vaginalis infections in other animals, including rhesus monkeys, rabbits, hamsters and rats, have been reviewed by Trussel (1947), Jirovec and Petru (1968) and Honigberg (1978b) and will not be dealt with here.

The most commonly employed infection routes are intravaginal, intra-peritoneal and subcutaneous; intravaginal infections in mice, rats, guinea pigs and hamsters are more reliably established if the animals are first induced into permanent oestrus with exogenous oestrogens (Honigberg, 1978b).

#### 1.15.2 Guinea pigs

The majority of studies on T. vaginalis infections in guinea pigs have used the intravaginal route. Soszka, Kazanowska and Kuczynska (1962)

found that inflammation of the vaginal epithelium occurred 2 to 75 h post-inoculation and was accompanied by a heavy purulent vaginal discharge containing trichomonads. The vaginal epithelium was heavily infiltrated by leucocytes which collected in spaces between the layers of cells. Cytological changes in the epithelial cells included nuclear enlargement and a peri-nuclear vacuolisation. In a similar study Szytykiel (1962) noted that T. vaginalis caused cell shape changes, multiple nucleation and cytoplasmic vacuolisation in the vaginal epithelium. Infections in pregnant guinea pigs may cause the abortion of macerated fetuses (Ginel, 1962). A detailed histological and histochemical study of vaginal T. vaginalis infections in guinea pigs was made by Skrzypiec (1975).

### 1.15.3 Mice

#### 1.15.3.1 Intravaginal infections

There have been few attempts to establish T. vaginalis infections in the mouse vagina (Honigberg, 1978b). Cappuccinelli, Lattes and Martinetto (1973) achieved 100 per cent infection rates in oestrogenised Balb/c and Swiss T mice; the infections lasted for 3 months and could be transferred from one female to another by intercourse with a common male mate. The infections caused dysplasia and superficial lesions of the cervical and vaginal epithelium.

#### 1.15.3.2 Intraperitoneal infections

The pathological changes in the abdominal organs of mice inoculated intraperitoneally with large numbers ( $10^6$  or more) of pathogenic strains of T. vaginalis have been characterised by Reardon, Ashburn and Jacobs (1961), Teras and Roigas (1966) and Gobert, Georges, Savel, Genet and Piette (1969). Typically, the infection causes an exudative peritonitis with the accumulation of ascitic fluid. Caseous masses consisting of numerous parasites and leucocytes adhere to the abdominal organs. The trichomonads commonly invade the abdominal organs starting with the liver and pancreas causing inflammation and subsequent necrosis. The invaded liver develops necrotic foci and the parasites are found palisaded on the



border between the necrotic and uninjured tissue. Death generally occurs 5 to 7 days post-inoculation.

The fine structural changes in the parasitised livers of infected mice were studied by Brugerolle et al. (1974). T. vaginalis was first seen in the sinusoids which became dilated and obstructed by numerous parasites, neutrophils, lymphocytes and macrophages. The trichomonads were amoeboid possessing many pseudopodia with which they closely adhered to the hepatocytes lining the sinusoids. These hepatocytes showed degenerative changes including a depletion of glycogen granules, an increase in lipid globules and vacuoles and dilatation of endoplasmic reticulum and mitochondria. The trichomonads lysed the hepatocytes and phagocytosed their contents, and then proceeded outwards into normal tissue leaving a necrotic zone in their wake. The cells surrounding the infected areas were normal except for an absence of glycogen. The authors suggested that T. vaginalis destroyed the liver tissue by a combination of active phagocytosis and by the secretion of toxins, the latter being responsible in part for the reduced glycogen content of the hepatocytes lying adjacent to the parasitised areas. Of interest in this study was the authors' discovery of microfilament meshworks in the pseudopodia of T. vaginalis, similar to those found in trichomonads adhering to the human vaginal epithelium (Nielsen and Nielsen, 1975); commenting on this finding, Brugerolle et al. suggested that the microfilaments formed part of an internal contractile system in T. vaginalis but they did not relate this system to the invasive and pathogenic behaviour of the parasite in the mouse liver.

#### 1.15.3.3 Subcutaneous infections

Subcutaneous infections in mice are of interest chiefly because they offer a reliable method of evaluating the inherent pathogenicity levels of strains of T. vaginalis. The "subcutaneous (SC) mouse assay" was developed by Honigberg (1961) and involves the SC inoculation of  $8 \text{ to } 9 \times 10^5$  T. vaginalis in 0.5 ml of an agar-containing medium into the flanks

of 6 week old C57/BL6J mice, although other strains of mice may be used (Jirovec and Petru, 1968). The relative pathogenicity of a strain of T. vaginalis is then obtained from the mean volume of the trichomonad-filled swellings protruding from the mouse flanks after 6 days. The pathology of the swellings or lesions was described by Frost and Honigberg (1962). The development of the lesions involves an orderly progression of events following the inoculation of the parasites; these events may be summarised, in sequence, as, (a) an influx of leucocytes into the infected area, (b) multiplication of the organisms, (c) destruction of the leucocytes and the surrounding host tissues, (d) oedema of the adjacent tissues and a second influx of leucocytes, (e) further multiplication of the parasites, which is followed by a continuation of the cycle. This progression leads to the formation of mantles of leucocytes and parasites lining the walls of a pus- and sometimes gas-filled lesion. SC infections are rarely fatal; with more pathogenic strains of T. vaginalis the lesions burst open after about 1 week and then heal, with milder strains the lesion may be walled off by a fibrous layer and the parasites degenerate. The severity of the lesions may be enhanced by treating the mice with cortisone before infection (Jeffries and Harris, 1967).

Using the SC mouse assay, Honigberg, Livingston and Frost (1966) and Kulda, Honigberg, Frost and Hollander (1970) were able to show that there was a good correlation between the level of pathogenicity of several strains of T. vaginalis in mice with the severity of the clinical and cytological findings in the female patients from which the organisms were isolated. Honigberg et al. (1966) studied 8 strains of T. vaginalis and found that the woman harbouring the strain least pathogenic for mice was an asymptomatic carrier of T. vaginalis; the woman harbouring the most pathogenic strain was found to have moderate vaginal discharge, cystitis, chronic cervicitis, marked inflammation of the cervical mucosa and carcinoma of the cervix. The other 6 strains which were of intermediate pathogenicity in mice were associated in the patients with vaginal and cervical



disease of corresponding intermediate severity. Kulda et al. (1970) obtained similar results to Honigberg et al. (1966) and they further noted that there was a positive correlation between the pathogenicity of 7 strains of T. vaginalis in mice and humans, and their rates of growth in axenic culture. The least pathogenic strain had a mean generation time in culture of 6 h; the most pathogenic, 13.5 h. As Kulda et al. (1970) pointed out, this discovery does not imply any causal relationship between high pathogenicity and slow growth since it is possible that the more pathogenic strains of T. vaginalis, living in a nutritionally-rich environment of pus, necrotic cells, many bacteria and tissue fluids, typical of severe vaginitis, may have become dependent on host growth factors and so adapt less well to growing in an artificial Trichomonas culture medium.

#### 1.16 Pathogenicity of T. vaginalis in cell cultures

##### 1.16.1 Introduction

There has been a widespread interest in the behaviour and pathogenicity of T. vaginalis in cell and tissue cultures since the pioneering studies of Hogue (1943, 1947). T. vaginalis-infected cell cultures, as models of trichomoniasis, offer the simplest and most direct way of studying the inherent cytopathogenic mechanisms of the parasite.

In most of the studies in this field T. vaginalis was inoculated into a culture vessel containing a continuous sheet or monolayer of cells on a glass coverslip. A wide range of cell types has been used including human embryonic epithelial cells, fibroblasts and myoblasts (Hogue, 1943; Kotcher and Hoogasian, 1957), WISH (human amniotic epithelium) cells (Dyner and Korbecki, 1974), HeLa cells (human adult cervical carcinoma) (Christian et al., 1963), monkey kidney fibroblasts (Kulda, 1967; Samoilescu et al., 1974), and chick embryo fibroblasts, hepatocytes and macrophages (Kotcher and Hoogasian, 1957; Farris and Honigberg, 1970). Despite this variety close similarities have been observed in the behaviour

of T. vaginalis towards the different cell types.

To date, almost all of the studies on T. vaginalis-infected cell cultures have been made with the aid of the light microscope; no detailed electron microscope studies have been published although in a brief abstract, devoid of particulars, Samoilescu et al. (1974) stated that they had examined the effects of the parasite on monkey renal cells using electron microscopy.

#### 1.16.2 Early studies

The first experimental infections of tissue cultures with T. vaginalis were described by Hogue (1943). She used primary explants of human embryo intestine, lung and muscle and chick embryo intestine. The infections caused granulation and vacuolisation of the cytoplasm of both epithelial and fibroblast cells; cell-free areas developed as a result of retraction of the cytoplasm and the cultures died within 24 h. Cell-free filtrates of "old" trichomonal cultures caused similar changes and so Hogue concluded that T. vaginalis destroyed the cells by producing toxins. Later Kotcher and Hoogasian (1957) studied infections in cultures of chick embryo cells, HeLa cells and human fibroblasts but although they noted cytopathological changes similar to those found by Hogue in her cultures, they considered that T. vaginalis caused the changes by mechanical means rather than chemical since they were unable to demonstrate any pathological effects of parasite-free T. vaginalis culture filtrates. Although these early studies were lacking in many details they served to stimulate further and more extensive studies the most significant of which were made by Christian et al. (1963), Kulda (1967) and Farris and Honigberg (1970); some of the details of these authors' studies are summarised in Table 2, which shows the specifications of the cell cultures, the details of the strains of T. vaginalis used, the size of the inoculum of parasites, the pathological changes which the parasites cause to the cultures, and the times taken for the parasites to destroy the cultures. As the table shows



direct comparisons of the results of the various studies on T. vaginalis-infected cell cultures are not easy due to the differences in the type of cells, in the relative pathogenicity of the strains of parasite, and in the sizes of the inocula of parasites used in the studies, nevertheless some salient features of the behaviour and pathogenicity of T. vaginalis towards cells are common to each of the various studies.

### 1.16.3 Behaviour of T. vaginalis in cell cultures

When a monolayer of cultured cells is infected with a population of T. vaginalis one of the first and most characteristic events is the affinity of the parasites for the cells; the organisms settle on and adhere to the cells within the first few hours of the infection (Hogue 1943). Kulda (1967) infected monkey kidney fibroblast cultures with  $4 \times 10^5$  T. vaginalis and noted that most of the organisms were adhering to the cells between 4 and 8 h post-inoculation. Some strains of T. vaginalis adhere less well to cell monolayers than others; Farris and Honigberg (1970) showed that the adhesiveness of a particular strain may be related to its pathogenicity level in humans. These workers infected embryonic chick liver cell cultures with two strains of T. vaginalis, JH30A and JH32A, the former was isolated from a woman with severe vaginitis and the latter from a woman with mild disease and only slight vaginal discharge; they found that at 2 h post-inoculation of the chick cell cultures with  $10^5$  parasites, 85 per cent of the more pathogenic JH30A strain were adhering to the cells compared to only 50 per cent of the milder JH32A strain; at 20 h there were still twice as many parasites of the pathogenic strain adherent to the cells as compared to the mild strain.

Following adhesion to cells T. vaginalis frequently becomes amoeboid and extends pseudopodia over the surfaces of the cells (Farris and Honigberg, 1970). Christian et al. (1963) used cinemicrography to study the interaction of T. vaginalis with monolayers of HeLa cells and noted amoeboid organisms moving above and beneath the monolayers and they concluded that

the activity of the parasites was responsible for the subsequent detachment of the HeLa cells from their glass substratum.

Both Hogue (1943) and Christian et al. (1963) observed an interesting behavioural characteristic of T. vaginalis in their cell cultures; they noted that the parasites frequently aggregated into large clumps comprising of many active organisms and they called this process "rosetting" (Hogue, 1943) or "swarming" (Christian et al., 1963). Although unsure of the cause of this behaviour Christian et al. (1963) speculated that the organisms were participating in a process of "genetic recombination". At present there is no evidence that T. vaginalis can pass on and/or exchange genetic material between individuals but clearly this possibility merits further study following the discovery of Honigberg, Livingston and Stabler (1971) that the exposure of a non-pathogenic strain (as measured by the SC mouse assay) of T. gallinae to the native DNA and RNA of a highly pathogenic strain of T. gallinae resulted in an enhanced pathogenicity of the former strain.

#### 1.16.4 Pathological changes in T. vaginalis-infected cell cultures

In general pathological changes are more evident in cells to which amoeboid T. vaginalis are adhering (Farris and Honigberg, 1970). These changes include retraction of the peripheral areas of the cytoplasm which leads to spaces appearing between adjacent cells, an increased vacuolation of the cytoplasm, and morphological abnormalities of the nuclei such as nuclear swelling and a condensation of the chromatin into granules. The gradual degeneration of the cells culminates in cell lysis. These changes are similar to those seen in the cells of the vaginal and cervical epithelium in natural T. vaginalis infections; this similarity is cogent evidence for the value of T. vaginalis-infected cell cultures as models of human trichomoniasis.

The cytochemical changes which T. vaginalis caused in chick liver cell cultures were extensively studied by Sharma and Honigberg (1966, 1967, 1969, 1971). They noted that typically the cells showed abnormally high levels of various



metabolic enzymes such as acid phosphatase and non-specific esterase and there was a large accumulation of lipid granules in the cells, and the authors considered that these and the other cytochemical changes which they found in the cells reflected a general decrease in metabolic activity and an increase in autophagocytosis which are changes which are not specifically related to the actions of T. vaginalis since they are also characteristic of diseased or degenerating cells in general.

As Table 2 shows, the times at which these changes are first seen after the inoculation of a cell culture with T. vaginalis may vary accordingly to the inherent pathogenicity of the strain of T. vaginalis used, and the size of the infecting inoculum. Christian et al. (1963) found that few pathological changes were elicited in HeLa cell cultures by an inoculum of 500 parasites and they likened this situation to an asymptomatic T. vaginalis infection in humans.

One of the features of T. vaginalis-infected cell cultures is that the pathological changes often occur not throughout the cell culture but in discrete foci with the result that the culture or monolayer of cells is dotted with cell-free areas where the cells have been lysed (Christian et al. 1963; Farris and Honigberg, 1970). These areas, or lesions, contain numerous parasites which adhere to the exposed glass substratum and to the cells lining the lesions. As the infections progress the lesions enlarge as the parasites migrate outwards lysing more cells. This feature suggests that a close association of the parasites with the cells is necessary for cell lysis since those cells lying in the intact areas of the cultures are often undamaged (Christian et al. 1963).

#### 1.16.5 Mechanisms of pathogenicity of T. vaginalis

Most of our understanding, albeit rather limited at present, of the inherent mechanisms of the cytopathogenicity of T. vaginalis has come from studies using cell cultures. Broadly there are two schools of thought; the first holds that the parasites secrete chemical factors into its environment which are potentially injurious to cells and tissues, and the second

proposes that the parasite mechanically damages cells as a consequence of its ability to adhere to them. It is by no means clear that either mechanism alone is responsible for the cytopathogenicity of T. vaginalis.

#### A. Chemical mechanisms

Hogue (1943) proposed that T. vaginalis secreted toxins when she found that parasite-free filtrates of "old" (sic) T. vaginalis cultures were capable of killing cell cultures in the absence of the organisms. Farris and Honigberg (1970) also found that filtrates of "actively growing" cultures of a strain of T. vaginalis, highly pathogenic in mice, produced pathological changes in chick liver cells which were qualitatively similar to those caused by the parasites (see Table 2) but the level of the changes and the number of cells affected was much lower. On the other hand no such pathogenic effects of culture filtrates on cells could be demonstrated by Kotcher and Hoogasian (1957), Christian et al. (1963) or Kulda (1967); these workers found that cell damage only occurred in the presence of the parasite. These apparently contradictory results may have arisen due to the differences in strains of T. vaginalis used and to differences in the states of the cultures from which the filtrates were obtained; there is clearly a need for more standardisation in experiments designed to test the effects of culture filtrates before this question can be resolved. It is possible that the putative toxic factors in filtrates may be very labile which might explain why the most extensive cytopathological changes are seen in those cells to which T. vaginalis is adhering (Farris and Honigberg, 1970), but as yet no one has been successful in identifying, isolating or characterising any potentially cytopathogenic substances from culture filtrates. Hogue (1943) found that filtrates of old T. vaginalis cultures did not digest gelatin and concluded that the cytopathogenicity of filtrates was not due to the presence of proteolytic enzymes.



## B. Mechanical mechanisms

It has been argued that because T. vaginalis characteristically adheres to cultured cells the parasite may be capable of mechanically injuring the cells' surfaces causing cell death but the ways in which the parasite may do so have not been fully investigated. Farris and Honigberg (1970) observed amoeboid organisms which were "pulling away" pieces of the cytoplasm of chick macrophages and at times the organisms ingested the pieces. Christian et al. (1963) also thought that it was the "intense activity" of amoeboid T. vaginalis both on top of and underneath a monolayer of HeLa cells that caused the break-up of the monolayer and the death of the cells; since the amoeboid organisms were able to crawl over the HeLa cells' surfaces it is possible that the tensile forces generated during movement of the parasites could mechanically disrupt the cells' plasma membranes leading to a lethal leakage of cytoplasmic components. Such a possibility was suggested by Nielsen and Nielsen (1975) who found amoeboid T. vaginalis adhering to the surfaces of human vaginal epithelial cells.

The lack of any conclusive evidence that T. vaginalis can mechanically damage cells is to a large extent due to the fact that the intimate association of the parasite with cells has not been examined in detail at the fine-structural level, consequently at present we have little idea of what events are occurring at the sites of contact between T. vaginalis and cells.

### 1.17 Aims of this study

The purpose of this study is to fill some of the gaps in our knowledge of the behaviour and pathogenicity of T. vaginalis in cell cultures which I have pointed out in the preceding review of the literature. Particular attention is paid to the following topics:

- 1) The fine structure of the adhesions between T. vaginalis and epithelial cells.
- 2) The fine structural changes caused within epithelial cells by the parasite.

- 3) Scanning electron microscopic examination of T. vaginalis.
- 4) The amoeboid movements of T. vaginalis.
- 5) The mechanism of adhesion and amoeboid motility in T. vaginalis.
- 6) The role of hydrolytic enzymes in pathogenicity of T. vaginalis.
- 7) Possible mechanisms of pathogenicity of T. vaginalis in experimental infections and in humans.



## 2. MATERIALS AND METHODS

### 2.1 Culture media and culture techniques

#### 2.1.1 T. vaginalis culture medium

The culture medium used for the primary isolation of T. vaginalis and for its maintenance in the laboratory was that described by Lumsden et al. (1966) with the modifications detailed below. This modified medium is hereafter referred to as Lumsden's Trichomonas Medium (LTM). The recipe is given below; AnalaR reagents and distilled water were used throughout.

#### Recipe for 1 litre of LTM

Neutralised Liver Digest (Difco Ltd), 40g per l.	300	ml
Dextrose, 0.308 M	100	ml
Calf serum (Wellcome Ltd)	100	ml
Salts solution:	392.5	ml

Prepared by mixing the following 4 solutions in the ratio indicated:

NaCl	0.154 M	100	} 108 vols
KCl	0.154 M	4	
MgCl <sub>2</sub>	0.103 M	3	
CaCl <sub>2</sub>	0.103 M	1	

Phosphate buffer pH 6.8	100	ml
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Prepared by mixing the following 2 solutions in the ratio indicated:

NaH <sub>2</sub> PO <sub>4</sub>	0.154 M	4.13	} 10 vols
Na <sub>2</sub> HPO <sub>4</sub>	0.103 M	5.87	

Bromocresol purple	15	mg
Sodium thioglycollate	1	g
Benzyl penicillin (Glaxo Ltd), 200,000 U / ml	5	ml
Streptomycin sulphate (Glaxo Ltd), 200,000 U / ml	2.5	ml
Nystatin (Squibb Ltd)	0.25	g

The salts solution and the buffer component were made up separately. The ingredients were mixed at room temperature and the final pH of the medium was checked and adjusted to 6.8 if necessary with 1M HCl. 4 litres of

medium were made up at one time and then sterilised by Seitz filtration and dispensed into sterile 15 ml, 100 ml, 200 ml and 500 ml screw-capped bottles. The 15 ml bottles were stored at 4°C and the larger volumes at -20°C. The bottles were filled to the brim to exclude as much air as possible to maintain the anaerobic conditions of the medium. 15 ml bottles of LTM were fitted with centrally-perforated screw caps with white rubber wads to allow inoculation and sampling of the culture medium with a syringe and needle through the rubber wad after the wad had been sterilised by wiping with 70 per cent alcohol. Nystatin was added to the medium after filtration because of its particulate nature. LTM containing Nystatin was only used for the primary isolation of T. vaginalis; for laboratory maintenance of trichomonads Nystatin was omitted.

The modifications to the recipe of Lumsden et al. (1966) for LTM were:

- 1) The replacement of liver digest with neutralised liver digest.
- 2) The alteration of the pH of the buffer component from 7.4 to 6.8.

These changes gave a final pH of 6.8 to the medium compared to 6.1 to 6.4 with the original formulation of Lumsden et al. Heath (1972) showed that T. vaginalis grows equally well in culture media with initial pH values of 6.4 and 6.8.

#### 2.1.2 Maintenance of T. vaginalis in culture

For raising large numbers of parasites for use in experiments, T. vaginalis was removed from cryopreservation (see 2.3) and grown in LTM. One or two glass capillary tubes, diameter 1 mm, of a selected stock of T. vaginalis were removed from liquid nitrogen storage and thawed in tap water at room temperature. The tubes were wiped with 70 per cent alcohol, the ends were broken off and the contents were expelled into a 15 ml bottle of LTM prewarmed to room temperature. The inoculated cultures were incubated at 37°C until the organisms had multiplied to a density of



about  $10^5$  per ml or more which generally took 2 to 3 days. At this stage the trichomonads were subcultured by withdrawing 0.5 to 1 ml of medium through the rubber wad of the culture bottle and transferring the organisms to a fresh bottle of LTM prewarmed to  $37^\circ\text{C}$ , again through the rubber wad.

Parasites for use in experiments were always taken from the first subculture post-thawing of cryopreserved stocks. Only cultures in the logarithmic phase of growth were used, i.e. between  $5 \times 10^4$  and  $10^6$  organisms per ml (Heath, 1972). As the density of organisms increases in the cultures the acidity of the medium increases due to their production of lactic acid; consequently a change in the colour of the bromocresol purple in the LTM, from purple to yellow, indicates an infected culture. The presence of trichomonads can then be checked by microscopy of a sample of the culture medium.

Organisms were harvested by centrifugation at 250 g for 10 min. Counts of parasite density were made either with a haemocytometer or on a Coulter counter model B.

### 2.1.3 Epithelial cell culture medium

RK 13 epithelial cells were grown in Medium 199, hereafter abbreviated to M199.

#### Recipe for 100 ml Medium 199

Medium 199 (Wellcome Ltd)	90	ml
Foetal calf serum (Wellcome Ltd)	10	ml
HEPES buffer 1 M (Wellcome Ltd)	2.5	ml
Benzyl penicillin (Glaxo Ltd) 100,000 U / ml	0.1	ml
Streptomycin sulphate (Glaxo Ltd) 50,000 U / ml	0.1	ml
Amphotericin B (Squibb Ltd) 2.5 mg/ml	0.1	ml

The final pH of M199 was 7.2. The medium was stored at  $-20^\circ\text{C}$ .

### 2.1.4 Maintenance of epithelial cell cultures

RK 13 cells were grown on one side of flat sided 50 or 100 ml sterile

screw capped glass bottles in 10 ml M199, and incubated at 37°C. The medium was changed every 2 days. Cells were passaged weekly. The cells were removed from the glass bottles by adding 10 ml of a solution of 0.02 per cent w/v Versene (Wellcome Ltd) containing 0.25 per cent w/v trypsin (Wellcome Ltd). The cell monolayers were incubated for 10 min. at 37°C in this cell dispersant until the cells were just starting to detach from the glass; the dispersant was decanted and the cells suspended in fresh M199 (40 ml) and distributed into fresh bottles. By using a split ratio of 1:4 (i.e. the cells from one bottle distributed into four bottles) the epithelial cells formed a confluent monolayer within 3 to 4 days.

For use in experiments the cells were grown on a variety of glass substrata:

- 1) For preliminary light microscopical studies the cells were grown on 9 x 35 mm coverslips in Leighton tubes with 1.5 ml M199 overlay.
- 2) For transmission electron microscopy the cells were grown on 32 mm diameter round glass slides in 60 ml screw-capped plastic specimen jars (Sterilin Ltd) with 5 ml M199 overlay.
- 3) For scanning electron microscopy the cells were grown on 22 mm diameter coverslips in jars as described in (2).

These cultures were incubated at 37°C with a daily change of M199 until a confluent monolayer of cells, with a density of about  $2 \times 10^3$  cells per  $\text{mm}^2$ , had formed which generally took 3 to 4 days.

## 2.2 Source, collection and isolation of T. vaginalis and epithelial cells

### 2.2.1 T. vaginalis

Trichomonas vaginalis was isolated from new female patients attending the clinic at James Pringle House, Middlesex Hospital, London W1. The isolation technique used was modified from that of Robertson et al. (1969) and has been described by Ackers et al. (1975).

The physicians at the clinic were asked to collect vaginal secretions from all new female patients during the period of examination of the



patient. Sterile pieces of polyester sponge 20 x 5 x 5 mm were held in a pair of Rampley's forceps and inserted into the posterior fornix of the vagina and allowed to soak up any secretions present, then withdrawn and placed directly into a sterile screw-capped 5 ml bijou bottle. The bottles were labelled with the patient's code number and stored at room temperature until they were collected and brought to the Department of Medical Protozoology for processing. Specimens were collected twice daily at 12 noon and 4 p.m. thus each specimen reached the laboratory within 4 h of collection from the patient. Preliminary experiments had shown that T. vaginalis remain viable in these conditions for up to 24 h.

In the laboratory 1 ml of sterile saline was added to the sponge and the bottle was then attached to the top socket of a sterile Hemmings filter apparatus (R.B. Turner, East Finchley, London) which was without a filter pad and which carried an empty 5 ml bijou bottle on the lower socket. The assemblies were centrifuged at 250 g for 10 min; during the centrifugation the sponge was compressed against the central perforated plate of the Hemmings apparatus and its contents were expelled into the lower bottle, collecting as a deposit and a clear supernatant. The supernatant was removed with a pasteur pipette and stored at -20°C for immunological analysis (Ackers et al., 1975).

The deposit was suspended in 0.2 ml of LTM and inoculated through the cap into a 15 ml bottle of LTM and incubated at 37 C. A small sample of the deposit, approx 0.01 ml, was retained and placed on a microscope slide under a coverslip to make a wet preparation for an immediate microscopical examination for the presence of T. vaginalis.

The wet preparations were examined under the 40x phase contrast objective of a Wild light microscope and a record was made of the presence or absence of T. vaginalis, epithelial cells and leucocytes, using the following arbitrary scale:

For T. vaginalis: 0 = no trichomonads seen; 1 = 1 trichomonad per field;

2 = 1 to 2 per field; 3 = 3 to 4 per field; 4 = 4 per field.

For epithelial cells and leucocytes: 1 = a few cells per field; 2 = many cells per field; 3 = cells almost confluent.

After examination the coverslips were removed and the wet films were allowed to dry at room temperature, and stored for reference.

The inoculated cultures were incubated for 7 days and examined daily for indicator colour change signifying an infected culture. The presence of trichomonads in cultures changing colour was confirmed by light microscopy of a sample. Most positive cultures were detected within 3 days; cultures still negative after 7 days as judged by no colour change were discarded.

The trichomonads were harvested in the late logarithmic or stationary phase of growth at a density of between  $7.5 \times 10^5$  and  $1.5 \times 10^6$  cells per ml (Heath, 1972) by centrifugation at 250 g for 10 min. The pelleted organisms were resuspended in 1 ml of fresh LTM and cryopreserved for subsequent use. (see 2.3).

T. vaginalis was isolated from 42 vaginal secretions out of 508 examined in the above manner. Some clinical and laboratory details of each stock of T. vaginalis are given in Table 3.

The two main modifications that were made to the isolation technique described by Robertson et al. (1969) and which were used in this study were:

- 1) The use of smaller pieces of sponge, 20 x 5 x 5 mm compared to 40 x 10 x 10 mm, which were easier to fit into the 5 ml bijou bottles of the Hemmings apparatus.
- 2) The inoculation of the whole deposit into one 15 ml bottle of LTM instead of two 5 ml bijou bottles, in order to have larger numbers of organisms in positive cultures for cryopreservation.

#### 2.2.2 RK 13 epithelial cells

RK 13 rabbit kidney tubule epithelial cells were obtained from Gibco



BioCult Ltd., Glasgow.

### 2.3 Cryopreservation of *T. vaginalis*

The majority of the stocks of *T. vaginalis* were cryopreserved for use in experiments at a later date. Cryopreservation preserved the inherent level of pathogenicity of *T. vaginalis* as judged by the severity of infections in mice (Ivey, 1975).

The technique used is a routine procedure in the Department of Medical Protozoology, LSHTM, and was developed by Lumsden, et al. (1966) and Lumsden, Herbert and McNeillage (1973). A suspension of *T. vaginalis* in fresh LTM was cooled to 0°C in an ice bath. 0.2 ml of the suspension was mixed with 0.8 ml of 12.5 per cent v/v dimethyl sulphoxide in LTM. This mixture was then dispensed into sterile capillary tubes which were then sealed at each end with a microburner. The volume of suspension in each tube was about 20 µl. The tubes were placed in methanol at room temperature and the methanol was then cooled on dry ice to -79°C in an insulating jacket of polystyrene for 24 h and then stored in liquid N<sub>2</sub> at -196°C.

Each stock of *T. vaginalis* was given a LUMP (London University Medical Protozoology) code number and the details of the stock, i.e. date of isolation, length of time in culture before cryopreservation, concentration of organisms in the tubes and the total number of tubes frozen, were recorded (Table 3).

### 2.4 Infection of RK 13 epithelial cell cultures with *T. vaginalis*

#### 2.4.1 Stocks of *T. vaginalis* used

In all 42 separate stocks of *T. vaginalis* were available for use in these experiments but since I had neither the time nor the resources to test each stock for its pathogenicity in epithelial cell cultures, only the following randomly-selected stocks were used: LUMP Nos 866, 873, 889, 896. No qualitative differences were found in the behaviour of these four

stocks in the cell cultures and for this reason the data obtained from experiments with these stocks of T. vaginalis have been pooled. The relative merits of this approach are discussed later (Section 4.1.2).

#### 2.4.2 Medium used in T. vaginalis-infected cell cultures

Preliminary experiments showed that T. vaginalis would not multiply in Medium 199 which was used for the maintenance of the epithelial cell cultures (see Christian et al., 1963, and Kulda, 1967, who also noted the inability of standard tissue culture medium to maintain growth of T. vaginalis). However a mixture of two parts LTM to one part Medium 199 was found suitable and did not have any adverse effects on either T. vaginalis or the epithelial cells over the experimental periods. The pH of the LTM/M199 mixture was 6.8.

#### 2.4.3 Procedure

The culture medium of 3 or 4 day old epithelial cell cultures was decanted and replaced with the LTM/M199 mixture culture medium containing  $2 \times 10^5$  T. vaginalis; the total volume of the culture medium depended on the vessel in which the cells were grown (see 2.1.4). The cultures were incubated at 37°C for the duration of the experiments. Parallel control cell cultures without T. vaginalis were set up using the LTM/M199 mixture or M199 alone as culture media. Generally 10 to 20 epithelial cell cultures were infected with T. vaginalis in any one experiment and these were fixed at varying intervals from 1 to 36 h post-infection for microscopical examination. Changes in the pH of the medium of the infected cultures over the experimental period of 36 h were monitored by measuring the pH of the decanted medium from cultures fixed for microscopical study.

### 2.5 Light microscopical techniques

#### 2.5.1 T. vaginalis-infected cell cultures

##### 2.5.1.1 Living cultures

Living T. vaginalis-infected cell cultures were removed from the



incubator and observed for a few minutes on an inverted microscope with phase contrast optics. For the first 6 h of infection, observations were made every 30 min; from 6 h until 36 h post-infection, every 2 to 3 hours. The start-times of experiments were staggered so that all phases of the infections could be examined during normal working hours.

#### 2.5.1.2 Fixed cultures

The cell cultures were fixed for light microscopical examination at varying intervals. The fixation procedure was as follows:

- 1) The culture medium was carefully decanted from the vessels.
- 2) The cultures were fixed in either absolute methanol for 1 min. or in 5 per cent v/v glutaraldehyde in 0.066 M sodium cacodylate buffer pH 7.2, for 5 min. Both fixatives gave satisfactory preservation of detail.
- 3) The cultures were stained in 10 per cent Giemsa stain in phosphate buffer, pH 7.2 for 10 min., air dried and embedded in Euparal Vert mounting medium on a slide.

Photomicrographs were taken on Kodak Pan-X 35 mm film using a Zeiss photomicroscope.

### 2.5.2 Studies on the morphology and motility of *T. vaginalis* on glass coverslips.

#### 2.5.2.1 Observation chamber

An observation chamber was constructed from two coverslips placed either side of a 20 mm diameter hole in a stainless steel slide 75 x 35 x 1 mm. A 32 mm<sup>2</sup> glass coverslip was fixed over one side of the hole with silicone grease. A suspension of *T. vaginalis* in LTM, containing approximately  $1 \times 10^4$  organisms per ml, was placed in the chamber. A second coverslip was sealed over the exposed side of the hole with paraffin wax, taking care to exclude air bubbles to ensure anaerobic conditions. The completed chamber was incubated horizontally at 37°C for 4 h to allow the trichomonads to settle on and attach to the coverslip on the lower side of

the chamber.

#### 2.5.2.2 Photomicroscopical techniques

The observation chamber was removed from the incubator, inverted and placed on the stage of a Zeiss photomicroscope which was equipped for phase contrast, Nomarski and interference reflection light microscopy. The chamber was maintained at 37°C with an air curtain incubator.

Phase contrast and Nomarski differential interference contrast micrographs of the morphology and motility of T. vaginalis were taken of those organisms which remained adherent to the upper coverslip of the chamber after inversion.

The attachments to the Zeiss photomicroscope used for interference reflection (IR) microscopy consisted of a mercury-arc epi-illuminator from which the 546 nm line was isolated with a filter. A 40x oil immersion objective, numerical aperture 1.0, was used.

As the motility of the trichomonads led to distortion of the image during exposure, the parasites were fixed before IR photography. The chamber was disassembled and the coverslip to which the trichomonads were adherent was immersed in glutaraldehyde fixative (see 2.6.1.1) at room temperature for 10 min. The chamber was reassembled and filled with cacodylate buffer.

Photomicrographs were taken on Kodak Pan-X 35 mm film.

### 2.6 Electron microscopical techniques

#### 2.6.1 Transmission electron microscopy (TEM)

##### 2.6.1.1 Standard fixation and embedding procedure

The culture medium of the T. vaginalis-infected cultures was carefully, so not to dislodge any trichomonads, decanted from the incubation vessels and the cultures were processed for TEM as follows:

- 1) Fixed in 3 per cent v/v glutaraldehyde in 0.066 M sodium cacodylate buffer pH 7.2 at 4°C for 1 h.



- 2) Washed in cacodylate buffer for 30 min. at 4°C.
- 3) Post-fixed in 1 per cent osmium tetroxide plus 4 per cent sucrose in cacodylate buffer at room temperature for 1 h.
- 4) Washed in buffer for 10 min.
- 5) Stained in 0.5 per cent uranyl acetate in 10 per cent ethanol for 30 min.
- 6) Dehydrated in ascending concentrations of ethanol, 50, 70, 80, 90, and 100 per cent, 10 min. each.
- 7) Washed in toluene 30 min.
- 8) Embedded in Araldite and polymerised for 2 days at 60°C.

The same procedure was used for TEM of uninfected epithelial cell cultures and axenic cultures of T. vaginalis. Monolayers of RK 13 cells were embedded in situ. Suspensions of trichomonads were embedded in 1 per cent agar after fixation and then handled as tissue pieces through the subsequent processing.

After polymerisation the embedded cultures were examined on a light microscope; sometimes phase contrast micrographs were taken of the embedded cells and trichomonads before sectioning. Areas of the cell monolayer selected for EM were removed from the glass substratum by boring around the selected area with a microscope attachment described by Bird and Chapman (1977). The resulting plug of Araldite containing the cells was easily detached from the coverslip by cooling the plug with solid CO<sub>2</sub>. The Araldite plug was glued to a perspex rod with cyanoacrylate adhesive and placed on an ultramicrotome.

Gold and silver coloured sections, 50 to 80 nm thick, were cut from the blocks on a Huxley Mk I ultramicrotome using glass knives. The sections were picked up on formvar-coated 100 mesh, 3 mm copper grids; sections were stained with lead citrate (Venables and Coggeshall, 1965) for 5 min and then examined in either a Zeiss EM9AS or a AEI/GEC EM6B electron microscope at 60 kV..

### 2.6.1.2 Ruthenium red (RR) staining

Some infected cell cultures were stained with RR prior to TEM. The fixation procedure was as above (2.6.1.1) except that 0.01 per cent v/v ruthenium red was included in both the glutaraldehyde and osmium fixatives.

The effects of a preliminary washing in salts solution and of neuraminidase on the staining of T. vaginalis with RR was tested in the following way. T. vaginalis was harvested from culture and washed 4 times by centrifugation and resuspension in sterile salts solution (for recipe see 2.1.1). The final pellet of T. vaginalis was resuspended in 6 ml of phosphate buffered 0.9 per cent sodium chloride, pH 7.2, and divided into 3 aliquots of 2 ml, each containing  $1.2 \times 10^7$  organisms, which were then treated in one of the following ways:

- 1) Incubated with 50 U per ml of neuraminidase (BDH Ltd) at 37°C for 30 min.
- 2) Incubated as (1) without enzyme.
- 3) Untreated.

At the end of this period the trichomonads were checked for viability using motility as the criterion; in each of the 3 aliquots more than 99 per cent of the cells were motile. The organisms were then centrifuged at 500 g for 5 min and the pelleted trichomonads were fixed and processed for transmission electron microscopy (see 2.6.1.1) with the inclusion of 0.01 per cent v/v ruthenium red in both glutaraldehyde and osmium fixatives.

### 2.6.1.3 Cytochemical localisation of acid phosphatase in T. vaginalis

T. vaginalis-infected cultures of RK 13 cells were processed for the cytochemical localisation of acid phosphatase using the following method (Venkatesen, 1973):

Solutions used:

1. 0.2 M acetate buffer pH 4.85.
2. Substrate medium:

0.2 M acetate buffer pH 4.85



Cytidine monophosphate ( $C_5MP$ ) (BDH Ltd)	10 mg (omitted in controls)
2 M sucrose	1.0 ml
0.79 M $MnCl_2$	0.5 ml
0.075 M $PbNO_3$	0.6 ml

Add reagents in order. Filter and preincubate for 30 min at 37°C before use.

3. 3 per cent glutaraldehyde in 0.066 M sodium cacodylate buffer pH 7.2.
4. Pre-incubation washing buffer: 0.066 M cacodylate +0.25 M sucrose, pH 7.2.
5. Post-incubation washing buffer: 0.5 M acetate +0.25 M sucrose, pH 4.85.
6. 1 per cent tetroxide in 0.066 M cacodylate buffer containing 4 per cent sucrose pH 7.2.

#### Procedure:

1. Fix the cultures in glutaraldehyde at 0°C for 30 min.
2. Rinse in pre-incubation washing buffer at 0°C for 10 min.
3. Incubate in the substrate medium for 30 min at 37°C.
4. Rinse in acetate buffer at 0°C for 30 min.
5. Post-fix in osmium for 1 h at 0°C.
6. Dehydrate in ascending concentrations of alcohol and embed in Araldite (see 2.6.1.1).

Controls were incubated in the substrate medium without the substrate  $C_5MP$ .

#### 2.6.2 Scanning electron microscopy (SEM)

The preparation and fixation of the infected cell cultures for SEM was as for TEM up to step 4 (see 2.6.1.1). After washing in buffer the cultures were dehydrated in ascending concentrations of acetone, 50, 70, 80, 90, 100 per cent, 10 min each. The cultures, in 100 per cent acetone, were then placed in a critical point drying apparatus, infiltrated with liquid carbon dioxide and critical point dried (Anderson, 1951). The coverslips were then broken into smaller pieces approximately  $10mm^2$  in

area, glued to stubs, coated with a 40 nm thick layer of gold and examined in a Cambridge Stereoscan S4.10 scanning electron microscope operating at 20 kV at a tilt angle of 45°.

## 2.7 Enzyme assays on *T. vaginalis*

### 2.7.1 Introduction

Qualitative and semi-quantitative biochemical assays were performed on homogenates of *T. vaginalis* in order to detect the presence of the enzymes acid phosphatase,  $\beta$ -glucuronidase and neuraminidase in the cytoplasm of the organism.

### 2.7.2 Preparation of homogenates of *T. vaginalis*

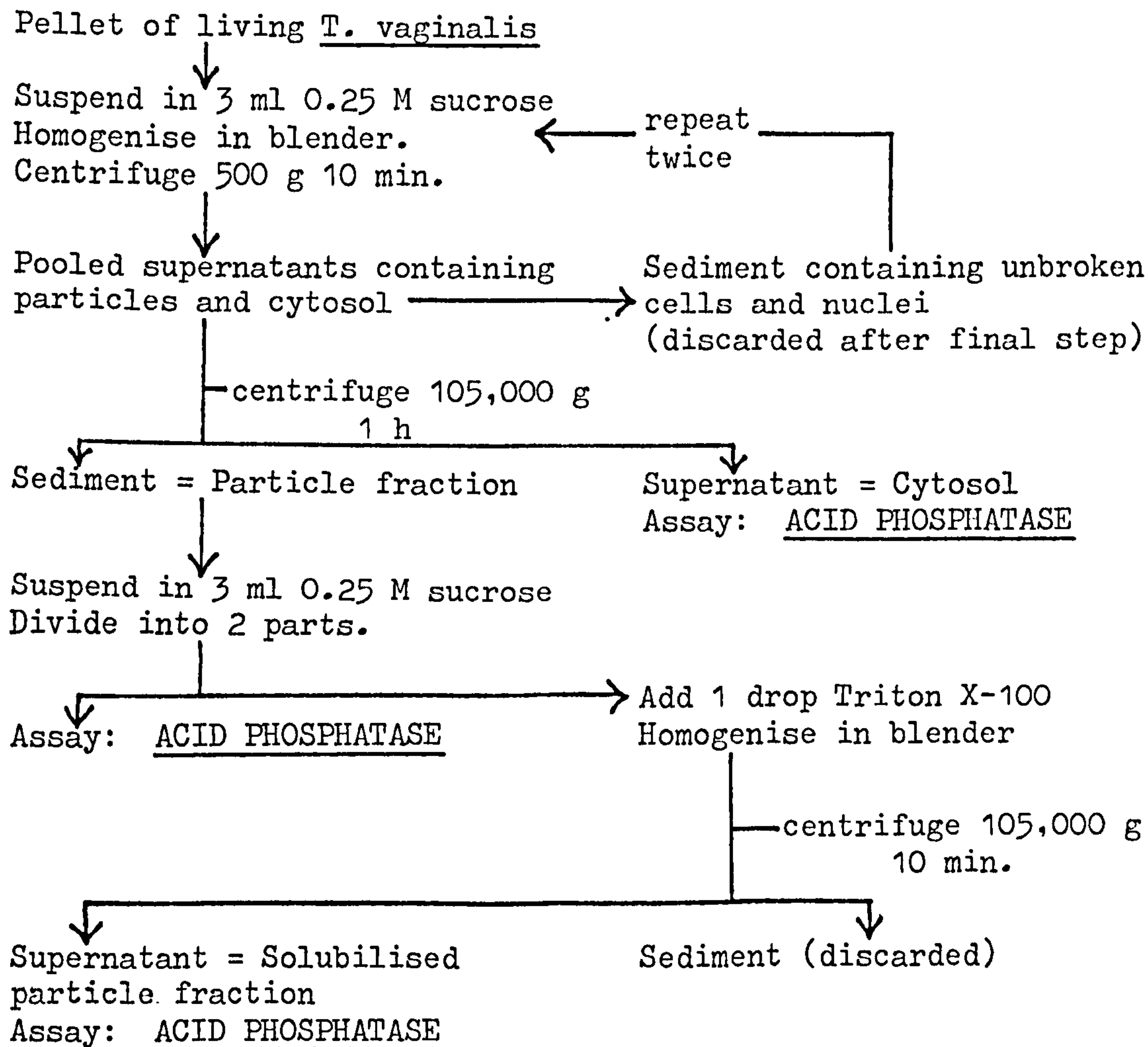
Pellets of living organisms were obtained by centrifugation from LTM cultures of *T. vaginalis*; the pelleted organisms were resuspended in either 0.25 M sucrose or in a buffer appropriate for the particular enzyme assay (see below). The various cytoplasmic fractions of *T. vaginalis* assayed for enzyme content, and the methods used to prepare them are set out in the flow charts below (2.7.2.1). The organisms were lysed either by rapidly freezing the suspension to -79°C in a bath of methanol containing solid CO<sub>2</sub> followed by rapid thawing to room temperature, and/or by homogenisation in a Waring blender equipped with a Teflon pestle at 300 rpm for 2 min. at 0°C.

Counts of organism density in the pellets were made with a haemocytometer. The protein concentration of some of the cytoplasmic fractions was determined by a Folin assay (Lowry, Rosebrough, Farr and Randall, 1951).

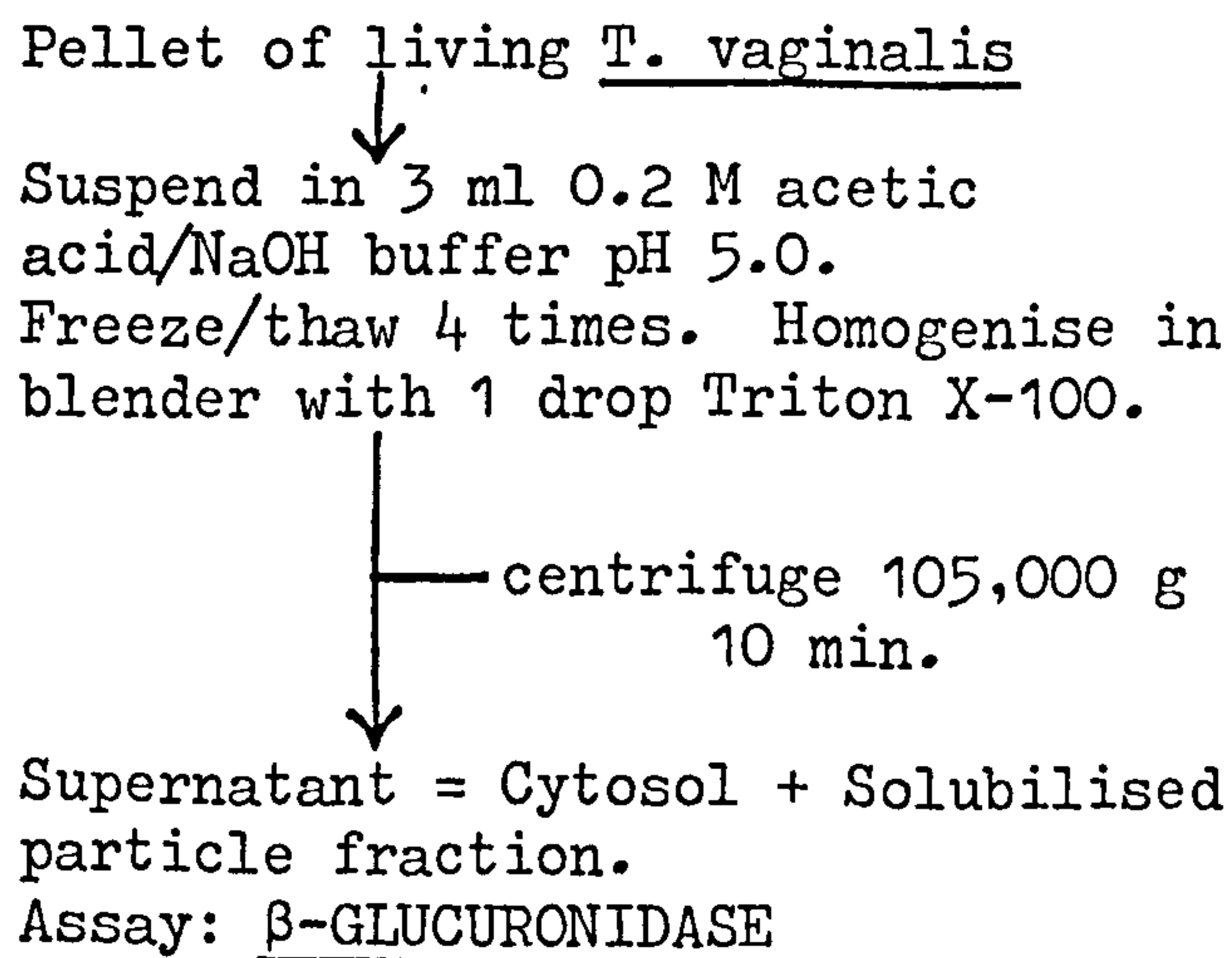


2.7.2.1 Flow chart showing the sequence of techniques used to obtain cytoplasmic fraction of T. vaginalis, and the enzyme assays performed on each fraction.

A. Acid phosphatase:



B.  $\beta$ -Glucuronidase



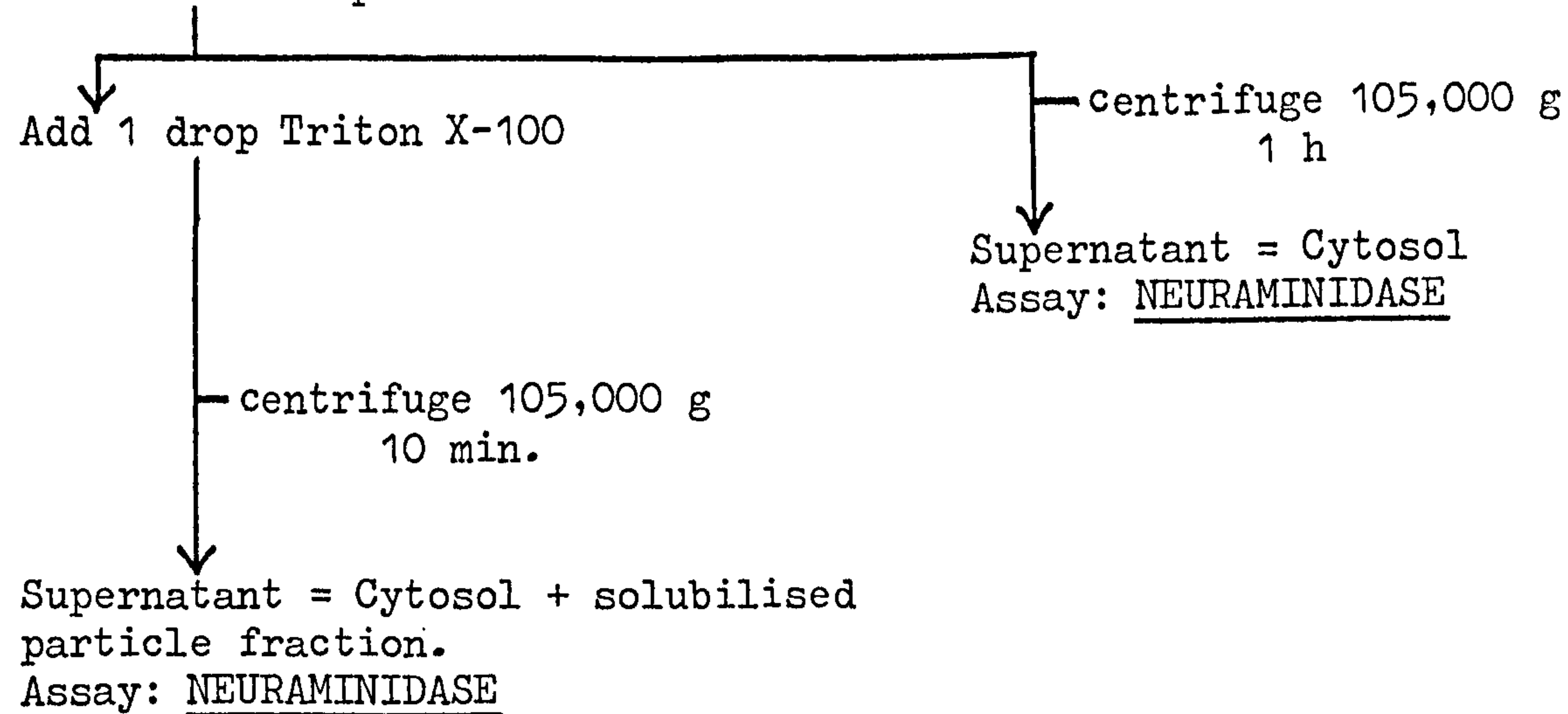
C. Neuraminidase

Pellet of living T. vaginalis

↓  
Suspend in 3 ml 0.2 M acetate  
buffer pH 6.1

Freeze/thaw 4 times

Divide into 2 parts





### 2.7.3 Assay for acid phosphatase (Venkatesan, 1973)

Solutions used:

1. 0.25 M sucrose.
2. 0.2 M acetate buffer pH 5.0.
3. 0.4 M glycine/NaCl/NaOH buffer pH 10.0.
4. 0.025 M paranitrophenol-phosphate (Pnp) (Calbiochem Ltd.) in 0.2 M acetate buffer pH 5.0.
5. 5 per cent trichloroacetic acid.
6. The T. vaginalis fraction to be assayed for acid phosphatase.

Procedure:

1. 0.5 ml of fraction was added to 0.5 ml Pnp solution and incubated in a water bath at 37°C for 10 min.
2. 2 ml of 5 per cent trichloroacetic acid was added to terminate the reaction.
3. 1 ml was removed from the reaction tube and added to 2 ml of glycine buffer.
4. The absorption at 435 nm was determined on a spectrophotometer. The results were compared with a standard curve constructed from the absorbances of a set of standards of known concentrations of paranitrophenol ranging from 20 to 100 nM.
5. A set of controls was run simultaneously: a) with T. vaginalis fraction replaced with 0.5 ml of sucrose solution, and b) with the Pnp solution replaced with acetate buffer.

### 2.7.4 Assay for $\beta$ -glucuronidase (Fishman, 1974).

Solutions used:

1. 0.2 M acetic acid/NaOH buffer pH 5.0.
2. 0.04 M para-nitrophenol- $\beta$ -D-glucuronide. (PnpG)
3. 0.4 M glycine/NaCl/NaOH buffer pH 10.0.
4. A solution of the T. vaginalis fraction to be assayed for  $\beta$ -glucuronidase.

Procedure:

1. 0.5 ml of the fraction was added to 1 ml acetic acid/NaOH buffer pH 5.0, 0.5 ml of PnpG and 2 ml of water.
2. The mixture was incubated at 37°C for 2 h and then the reaction terminated by the addition of 2 ml glycine buffer.
3. 2 ml samples were removed and the absorbance at 435 nm was read in a spectrophotometer and the results compared with a standard curve constructed from the absorbances of a set of standard solutions of paranitrophenol ranging from 20 to 100 nM.
4. A set of controls was run simultaneously a) without T. vaginalis fraction, b) with T. vaginalis fraction boiled for 10 min, c) without PnpG and d) without both fraction and PnpG. The latter was used to establish the baseline in the spectrophotometer.

2.7.5 Assay for neuraminidase and sialic Acid (Aminoff, 1961).

Solutions used:

1. 0.025 M periodic acid in 0.125 N  $H_2SO_4$  pH 1.2.
2. 2 per cent sodium arsenite in 0.5 N HCl.
3. 0.1 M 2-thiobarbituric acid, adjusted to pH 9 with 2 N NaOH.
4. Butan-1-ol containing 5 per cent 12 N HCl.
5. 0.2 M acetate buffer pH 6.1.
6. Collocalia sialomucoids (gift of Dr. J. P. Ackers) containing 10-12 per cent sialic acids.
7. Solution of T. vaginalis fraction to be assayed for neuraminidase

Procedure:

1. Into the incubation tubes was placed 0.5 ml of acetate buffer, 1 ml of an aqueous solution of 0.1 per cent w/v Collocalia sialomucoids and 0.5 ml of T. vaginalis fraction.
2. The mixture was incubated at 37°C for 30 min.
3. After incubation 0.5 ml was removed and added to 0.25 ml of periodate. This mixture was incubated for 30 min in a water bath at 37°C.



4. 0.2 ml sodium arsenite was added.
5. When the yellow colour of iodine had disappeared, 2 ml of thiobarbituric acid were added.
6. The mixture from 5 was placed in a boiling water bath for 7.5 min.
7. Cool in an ice-water bath for 5 min.
8. 5 ml acid butanol were added and the tubes shaken vigorously.
9. 2 ml of the acid butanol was removed and the absorption at 549 nm was read on a spectrophotometer. The molar extinction of the chromogen at 549 nm is  $70.7 \times 10^3$  and the extinction is directly proportional to the concentration of N-acetylneuraminic acid in the samples after incubation. The concentration of neuraminidase in the T. vaginalis fraction can thus be expressed in terms of the number of nmoles of N-acetylneuraminic acid released per min at 37°C.

### 3. RESULTS

#### 3.1 Light microscopical studies on *T. vaginalis*-infected RK 13 epithelial cell cultures

##### 3.1.1 Introduction

The general characteristics of the infection of 3-day-old RK 13 epithelial cell cultures with  $2 \times 10^5$  *T. vaginalis* were obtained from light microscopical observations on more than 80 living and fixed cell cultures. These studies enabled me to isolate the more important features of the infection which were subsequently examined in greater detail in the electron microscopical and morphology/motility studies that are described later (Sections 3.2, 3.3 and 3.4).

##### 3.1.2 Epithelial cell cultures - controls

The appearance of a typical 3-day-old culture of RK 13 epithelial cells is shown in Figure 2. After 3 days in culture the cells form a continuous sheet of polygonal cells; most of the cells had a single nucleus but bi- and multi-nucleate cells were sometimes present. Once the cells had formed a confluent sheet, or monolayer, multiplication of the cells ceased and no mitosing cells were seen. Many of the cells possessed empty cytoplasmic vacuoles, 5 to 30  $\mu\text{m}$  in diameter, which may have been a symptom of in vitro culture. The surface morphology and the fine structure of the epithelial cells are illustrated in Figures 19, 47, 48 and 49 and will be described later in the relevant sections.

##### 3.1.3 Epithelial cell cultures infected with *T. vaginalis*

The progression of the events which occurred following the inoculation of  $2 \times 10^5$  *T. vaginalis* into 3 to 4 day old epithelial cell cultures was seen to be made up of 5 sequential phases. The duration and the signs of these phases were:

Phase 1: 0 to 2 h post-inoculation; aggregation of *T. vaginalis* and adhesion of the organisms to the cell monolayer.



Phase 2: 2 to 6 h post-inoculation; first appearance of cell-free areas, or lesions, in the monolayer.

Phase 3: 6 to 10 h post-inoculation; enlargement of the lesions to about 10 per cent of the area of the cultures.

Phase 4: 10 to 24 h post-inoculation; coalescence of the lesions.

Phase 5: 24 to 36 h post-inoculation; cell monolayers totally destroyed.

The behaviour of the parasites and the pathological changes in the cell monolayer during each phase are described below.

#### Phase 1.

Within 30 min. of the addition of the parasites to the cell cultures, most of the organisms had settled out of suspension under gravity and were swimming actively over the upper surfaces of the cells. After one hour's infection many of the organisms aggregated into large clumps, 100 to 200  $\mu\text{m}$  wide, which comprised of up to 200 individuals (Fig. 3). It was clear that at this time the clumps were formed by the aggregation of many individuals, however at later stages of the infection aggregates may have arisen or increased in size following the multiplication of small groups of organisms, the resulting daughter organisms remaining within the parental clump. The aggregates were commonly surrounded by single organisms and smaller groups of trichomonads (Fig. 3). By this time the organisms, whether aggregated or not, lay almost motionless on the cell monolayer and gentle agitation of the culture vessel showed that they were sticking to the surfaces of the cells. However if the cultures were shaken vigorously the trichomonads could be detached from the cells; the cells on the other hand were not detached from the coverslips in this manner, showing that the adhesions of the cells to the glass coverslips were stronger than those of the parasites to the cells. Although the organisms were sticking to the monolayer, their flagella and undulating membranes were still actively beating in the culture medium. Sometimes the organisms were seen to be adhering to the

cells by their axostyles. During this first phase of the infection no morphological changes were seen in the cell cultures; the appearance of small areas of cell lysis marked the beginning of the second phase.

#### Phase 2.

After about 2 h infection the first signs of damage to the cell monolayer were observed. Small cell-free areas, or lesions, appeared beneath or to one side of the aggregates of organisms which adhered to the monolayer (Fig. 4). Initially these lesions were not more than 50 to 100  $\mu\text{m}$  wide but during the next 4 hours they doubled in width. No lesions were seen beneath smaller clumps or individual T. vaginalis and neither did the unparasitised areas of the monolayer contain lesions (Fig. 4). As the lesions widened so the aggregates of T. vaginalis flattened and spread on to the exposed glass coverslips, marking the third phase of infection.

#### Phase 3.

By 6 h infection most of the aggregates of T. vaginalis had flattened into lesions and had spread out leaving cell- and parasite-free areas of the substratum (Fig. 5). During this phase the monolayer was dotted with lesions and about 10 per cent of the area of the cell monolayers was destroyed by the trichomonads. By using high power phase contrast microscopy of fixed, unstained cultures it was possible to see in some detail what was occurring in the lesions.

Figures 6 and 7 shows typical lesions in the monolayer. The centres of the lesions were frequently free of cells and parasites. Most of the parasites were found pallisaded, sometimes two or three deep, against the edges of the cells bordering the lesions from where the parasites burrowed underneath the cells; the organisms were often seen lying either partly or totally beneath intact areas of the monolayer (Fig. 6). Many of the organisms were amoeboid in shape and closely flattened on the coverslips.

The enlargement of the lesions was due to the outward migration of the trichomonads lining the edges of the lesions; this migration occurred



mainly under the cell monolayer where the organisms had pushed themselves between the coverslip and the lower surfaces of the cells so lifting them away from the substratum. When relieved of some of their adhesions to the glass the normally polygonal epithelial cells often became elongated leaving long cytoplasmic processes, or retraction fibres, projecting out into the lesion (Fig. 7). This centripetal retraction of the epithelial cell cytoplasm was probably an important factor in the initial establishment of the lesions during phase 2; by causing the cells beneath them to retract, exposing the underlying substratum, the aggregates of T. vaginalis would have been able to flatten on the substratum and start migrating under the monolayer. As the parasites moved outwards they lysed the cells with which they were in contact; lysed cells often showed an increase in phase contrast of the cytoplasm and nuclei, and the latter were pyknotic (Figs. 6 and 7).

The unparasitised areas of the monolayer lying between the lesions did not appear affected by the trichomonads; pathological changes were restricted to the cells bordering the lesions and in contact with the parasites (Figs. 6, 7 and 8) suggesting a very local pathological effect of the organisms on the cell monolayer. The pH of the incubation medium at the end of Phase 3, i.e. about 10 h post-infection, had changed to 6.5 (from 6.8 at time 0 h) so it was unlikely that an increase in acidity of 0.3 units could have contributed to the lysis of the epithelial cells.

As the lesions grew, so adjacent lesions started to coalesce marking the next phase.

#### Phase 4.

From about 10 to 24 h post-inoculation the lesions continued to enlarge as the parasites moved outwards and left large areas of the coverslips devoid of cells (Fig. 8). The organisms removed the tracts of cells between adjacent lesions linking them up into larger and larger lesions. No differences in the behaviour of the parasites was noted in this phase;

they still adhered readily to both the cells and to the substratum, however there were also numerous organisms swimming freely over the lesions and intact areas of the monolayer and these were found in the decanted culture medium when the cultures were fixed for microscopy. By 24 h about 50 per cent of the cell monolayer was destroyed.

#### Phase 5.

During a period 24 to 36 h post-inoculation of the cell cultures with T. vaginalis the majority of the remaining cells were completely destroyed; only a few islets of cells remained attached to the coverslips, surrounded by many T. vaginalis (Fig. 9). The culture medium contained the debris of lysed cells and small sheets of abnormal cells. The damage to the cell cultures during this final phase was probably not due entirely to the action of the parasites but partly due to acidic conditions and medium exhaustion. In the majority of cultures the pH of the medium was 4.5 to 5.0 and the parasite density was  $1.0$  to  $2.0 \times 10^6$  organisms per ml.

#### 3.1.4 Summary of results

These results showed that when T. vaginalis is introduced into epithelial cell cultures the pathological changes in the cultures occur initially in small areas, i.e. the lesions, and that these damaged areas increase in size as the infection progresses. Thus the pathological effects of T. vaginalis on the cultures are discrete and do not affect all the cells at the same time. The two main factors in the formation of the lesions were the aggregation of the parasites and the adherence of the aggregates to the monolayer of cells, and to the coverslips once a lesion had formed. From then on the lesions enlarged by the destruction of the cells lining the lesions and with which the parasites were in contact. The epithelial cells lying within the intact areas of the monolayer between the lesions were unaffected by the presence of T. vaginalis in the cultures. The enlargement of the lesions was to a large extent due to the ability of the trichomonads to adhere to the glass coverslips and to migrate in an



amoeboid manner under the cells at the edge of the lesions. Therefore each lesion contained all the elements of the infection, which were the adhesion of T. vaginalis to other parasites, and to the cells and glass substratum, migratory amoeboid forms of T. vaginalis and finally epithelial cells undergoing various stages of attack from the parasites.

For these reasons, and to exclude as far as possible the effects of a changing acidity in the culture medium, the lesions produced after 6 h of infection were selected for further study by electron microscopy. For both the TEM and SEM studies, larger areas of RK 13 cell monolayers were used (see 2.1.4) but in both cases the standard inoculum of  $2 \times 10^5$  T. vaginalis was used. These small differences in the experimental techniques had no bearing on the behaviour of T. vaginalis or on the formation of lesions in the monolayers.

### 3.2 Scanning electron microscopy of T. vaginalis-infected RK 13 cell cultures.

#### 3.2.1 Introduction

Scanning electron microscopy (SEM) was used in order to understand the three-dimensional aspects of the interaction of T. vaginalis and epithelial cells. In particular this part of the study revealed the morphological specialisations of T. vaginalis as it adhered to and moved on the glass substratum and the cell monolayer and so complemented the motility studies which are described later.

#### 3.2.2 External morphology of amoeboid T. vaginalis

##### 3.2.2.1 Shape

In general the shape of the amoeboid T. vaginalis, whether adhering to the glass or to epithelial cells, was of two kinds, rounded with one large pseudopodium or elongated with several small pseudopodia. Figs. 10 and 11 illustrate the rounded shape; both organisms have a rounded anterior mass from which the flagella and undulating membranes project and which extends up into the medium away from the substratum. These trichomo-

nads have extended a single broad flat pseudopodium from their posterior poles and with which they adhere to the substratum. Although the upper (i.e. away from the substratum) surfaces of the pseudopodia of T. vaginalis were normally free of any surface projections their edges often possessed short thin projections, or filopodia, 0.5  $\mu\text{m}$  in diameter and between 2 and 5  $\mu\text{m}$  in length, which were attached to the substratum at their tips (Fig. 11). Fig. 12 shows the second type of shape which T. vaginalis adopted when adhering to the substratum. The Figure shows two elongated or polarised organisms, which are stretched out along their antero-posterior axes. Small pseudopodia project from the sides of the body, but in polarised organisms the pseudopodia and filopodia are mainly confined to the poles of the body (Figs. 12 and 28). Polarised organisms often had the central portion of the body lifted off the substratum and the catenary curves at the edges of these raised parts suggested that the organisms were stretched out between the adhesions of their peripheral parts to the substratum (Fig. 12). Filopodial extensions from amoeboid T. vaginalis were normally straight and since they were often only adhering to the substratum at their distal ends their straightness may have been due to tensions developed between the adhesive tips and the main body of the organism (Figs. 10, 11). Occasionally groups of several organisms adhered to the substratum forming confluent sheets with their pseudopodia (Fig. 13).

#### 3.2.2.2 External features

The plasma membrane of T. vaginalis is generally fairly smooth over the pseudopodia but more wrinkled over the body (Figs. 10, 11). Some of the wrinkling may be partly due to the collapse of the membrane on sub-membraneous structures during specimen preparation. Small pits, 0.1  $\mu\text{m}$  in diameter, were occasionally seen in the membrane and may be the openings of pinocytotic vesicles in the process of formation (Fig. 26).

Figures 14 to 17 show the arrangement of the anterior flagella and undulating membrane at the anterior pole of T. vaginalis. Hitherto these



structures have not been examined in detail by SEM. The four anterior flagella, the fifth recurrent flagellum and the undulating membrane (UM) emerge from the anterior pole in a shallow depression of the body which I shall refer to as the flagellar pocket (Fig. 15). This pocket is not always seen in TEM studies of cultured T. vaginalis (Nielsen et al., 1966) and so it is an open question whether the pocket is a permanent structure or only arises as a result of pseudopodial protrusion of the cytoplasm around the point of emergence of the flagella and UM.

The anterior flagella are 0.5  $\mu\text{m}$  in diameter and about 10  $\mu\text{m}$  long (Figs. 14, 17). The UM is a thin fold of cytoplasm about 1  $\mu\text{m}$  wide which takes a sinuous course as it passes posteriorly down the body. The recurrent flagellum is attached to the UM at a distance of 0.5  $\mu\text{m}$  from its free edge (Figs. 14, 15 and 16). The UM and recurrent flagellum pass backwards down the body of T. vaginalis and terminate together (Figs. 14 and 17).

The axostyle, which is so rarely seen completely in thin sections by TEM, projects from the posterior pole (Figs. 10, 32). It is normally about 5  $\mu\text{m}$  in length and 0.5  $\mu\text{m}$  in diameter when it emerges from the body; the axostyle tapers to a point in its distal portion.

### 3.2.2.3 Dividing forms of T. vaginalis

Some examples of trichomonads in various stages of division were observed in the infected cell cultures and these are shown in Figures 10, 17 and 18. One of the first signs of division seen was the presence of two sets of flagella and two UMs (Fig. 10). Figure 17 shows a late stage of division in which a cleavage furrow is present lying longitudinally between two daughter trichomonads. It is clear from these figures that the fact that the organisms were adhering to a substratum did not inhibit division. Future studies of the mechanisms of binary fission in T. vaginalis could take advantage of the ability of the organism to divide on a substratum; in this situation T. vaginalis could be fixed for fine structural examina-

tion at any stage of division which the observer chooses.

Figure 18 shows an abnormal dividing form of T. vaginalis; the organism has at least 5 separate sets of flagella. Such forms were rarely seen in cultured trichomonads and may represent degenerating T. vaginalis in which the mechanisms of cytokinesis are deficient. However this point merits further investigation.

### 3.2.3 External morphology of RK 13 epithelial cell cultures

In SEM the monolayer of RK 13 epithelial cells was seen as a confluent sheet of cells which were generally polygonal in shape and about 20 to 50  $\mu\text{m}$  in diameter (Fig. 19). The spaces between the cells were undoubtedly caused by the critical point drying process since they were not seen in the TEM studies (see Figs. 47, 48, 49). The dorsal surfaces of the cells are covered with many microvilli (Fig. 33).

No morphological differences were apparent between the control cell cultures incubated in the M199/LTM mixture and those incubated in M199 alone over a period of 6 h, i.e. during the time the T. vaginalis-infected cell cultures were incubated.

Uninfected monolayers of cells frequently contained smooth-surfaced spherical structures, about 3  $\mu\text{m}$  in diameter, which were also found in parasite-infected cultures (Figs. 12, 34); the nature of these objects could not be established.

### 3.2.4 Morphology of the lesions in T. vaginalis-infected RK 13 cell cultures

3 to 4 day old monolayers of epithelial cells were infected with  $2 \times 10^5$  T. vaginalis for 6 h and then fixed and the lesions produced in the monolayers were examined.

A typical lesion is shown in Fig. 20. The infected monolayers were dotted with lesions which were surrounded by large areas of complete monolayer to which a few aggregated and individual T. vaginalis were adhering (Fig. 20). Much of the central regions of the lesions were devoid of



epithelial cells (Figs. 21 and 22); the few isolated cells that remained in the centres of the lesions were often rounded up and possessed long cytoplasmic fibres which remained attached to the substratum as their peripheral cytoplasm retracted towards the cell centre (Figs. 21 and 31). The epithelial cells lining the edges of the lesions were frequently more rounded and projected further up into the medium than cells in the intact parts of the monolayer (Figs. 22 and 23) and some of the cells at the lesions' edges were lifted off the substratum (Fig. 22).

In contrast however, some of the isolated epithelial cells and those lining the edges of the lesions instead of retracting, extended broad flat lamellar processes over the cell-free areas of the lesions (Figs. 23, 24); this protrusive activity was presumably facilitated by the removal of neighbouring cells by the trichomonads so giving these cells extra substratum over which to spread. Thus it seems likely that the spreading of some of the epithelial cells was not a direct consequence of attack by T. vaginalis whereas the retraction of other cells may have been a sign of injury.

The majority of the trichomonads were found within the lesions; few organisms were seen on the intact areas of the epithelial sheet which lay around the lesions. Some of the organisms were adhering to the exposed substratum either singly (Figs. 10, 11 and 12) or in groups (Fig. 13) while others were adhering to the epithelial cells (Figs. 21, 22 and 23). Almost all of the parasites showed some degree of pseudopodial activity giving them an amoeboid morphology.

The exposed areas of the substratum in the centres of the lesions provided the trichomonads with a surface to which they could adhere and move on in an amoeboid manner. The protrusive activity of amoeboid organisms at the edges of the lesions appeared to be at least partly responsible for the lifting of epithelial cells away from the substratum. Figures 24, 25 and 26 show one such amoeboid T. vaginalis which has extended

a large pseudopodium under a cell; the cell is lying above the substratum where it may have been pushed as the organism moved under it.

Those T. vaginalis which adhered to the upper surfaces of the epithelial cells had a variable morphology; some were rounded (Fig. 27) and others were polarised (Fig. 28). Generally contact with the cells was made with the pseudopodia (Figs. 28, 32 and 33) leaving the body of the trichomonad and its flagella and undulating membrane projecting away from the cell (Figs. 29 and 30). Indeed, the flagella were rarely seen to be adhering to the cells; Figure 31 illustrates a single example in which the flagella of a parasite were looped around a retraction fibre of a cell. The axostyle of T. vaginalis was similarly not involved in the adhesion of the parasites to the epithelial cells (Fig. 32). The pseudopodia of T. vaginalis were normally in very intimate contact with the cells (Figs. 32 and 33) and sometimes enmeshed with the microvilli of the cell (Fig. 33); parasites such as those shown in Figs. 32 and 33 appeared to have been crawling over the surfaces of the cells at the time of fixation.

### 3.2.5 Pathological changes in the epithelial cells

It was obviously not possible to see what internal changes occurred within the cells by SEM but some surface changes were seen in the epithelial cells which may have been a sign of injury caused by the action of the trichomonads. One of these signs was the absence of microvilli on the surface of some cells which was only seen in cells to which T. vaginalis was adhering or lying adjacent to. Figure 34 shows two rounded epithelial cells one of which has lost most of its microvilli.

The surfaces of many T. vaginalis and of the epithelial cells in the lesions were covered with numerous vesicles and particles of variable sizes which were presumably released from lysed epithelial cells (Fig. 29). In Figure 34 the remains of a lysed cell are shown lying above a trichomonad. The full extent of the pathological changes within the cells is best seen in thin section by TEM and these changes are described in the next section.



### 3.3 Transmission electron microscopy (TEM) of *T. vaginalis*-infected epithelial cell cultures

#### 3.3.1 Introduction

The lesions produced in the RK 13 cell monolayers 6 h post-infection with *T. vaginalis* were ideally suited to a TEM study of the pathogenic mechanisms of the parasite. Because of the focal nature of the interaction of *T. vaginalis* and the monolayer, the many different stages of the interaction, from initial contact of trichomonad and cell to the lysis of the cells could be identified in each lesion. Figures 35, 36, 37, 38 and 39 illustrate the main fine structural features of *T. vaginalis* which were described in the Introduction (1.7). The following sections deal firstly with the fine structural specialisations seen at the points of contact between individual trichomonads and between the organisms and the substratum, and secondly with the fine structure of the contacts of *T. vaginalis* with the epithelial cells and the pathological changes that occurred in the cells as a result of their interaction with *T. vaginalis*.

#### 3.3.2 Specialised contacts between adjacent *T. vaginalis*

In the lesions the trichomonads were often tightly packed with large areas of their surfaces in close, and often parallel, apposition with a clear gap of about 50 to 100 nm between the apposed plasma membranes (Fig. 50). In some of these areas specialised contacts, which may have been sites of adhesion, were seen between adjacent trichomonads. These were chiefly of two types. The first consisted of the reciprocal interdigitation of the cytoplasm of two trichomonads. The interdigitations were formed from up to 10 thin pseudopodia, devoid of organelles but full of microfilamentous material, which made indentations up to 2  $\mu\text{m}$  long and 0.5  $\mu\text{m}$  wide in the cytoplasm of the opposite trichomonad. (Fig. 40). The microfilaments within the interdigitations were often organised into bundles 1 to 2  $\mu\text{m}$  in length which were oriented

perpendicularly to the opposite organism (Figs. 41 and 42). The apposed plasma membranes of the trichomonads were generally separated by a clear gap of 20 to 50 nm (Fig. 42).

The second type of contact was seen in regions of close and parallel contact between two organisms and did not involve the formation of pseudopodia. These adhesions were about 1  $\mu$ m in length; the apposed membranes were separated by a uniform gap of 75 nm. The extracellular space contained a central band of electron dense material lying parallel to the membranes which was traversed by filaments 5 to 10 nm thick which passed from one membrane to the other (Figs. 43 and 44).

### 3.3.3 Specialised contacts between *T. vaginalis* and the glass substratum

Much of the lower surface of many *T. vaginalis*, especially those lying beneath cells, was in close and parallel contact with the glass substratum (e.g. Fig. 51). The interface of the glass coverslips with the culture medium is marked by an electron-dense line, 5 to 10 nm thick, which probably consists of serum proteins adsorbed by the glass (Fig. 45). In some sections this line was absent, most likely because it remained attached to the glass when the Araldite blocks were detached (Figs. 51, 52 and 53). Nevertheless it was still possible even in these cases to see that the closeness of contact of *T. vaginalis* with the glass was similar to that between the cells and the glass. No cytoplasmic specialisations were noted when the central part of the body of *T. vaginalis* was lying in close contact with the substratum but the more peripheral parts which were generally in the form of pseudopodial extensions contained few cytoplasmic organelles except for a meshwork or matrix of microfilaments (Fig. 45). These pseudopodia were frequently lying within 30 nm of the substratum. The meshwork of microfilamentous material within the pseudopodia sometimes contained tracts or bundles of filaments oriented towards the tips of the pseudopodia where they



approached the substratum most closely (Figs. 45 and 46).

#### 3.3.4 Fine structure of the epithelial cell monolayer

Figures 47 to 49 illustrate the salient features of the epithelial cells within the monolayer before infection with T. vaginalis. The monolayer consists of a single sheet of cells with their lower surfaces closely applied to the substratum. The upper surfaces are covered with microvilli (Fig. 47). Small spaces occurred beneath the cells at the sides of thin lamellar cytoplasmic extensions (Fig. 48). The medium facing sides of the cells were joined laterally by tight junctions; intermediate-type junctions, commonly associated with an interdigitation of the adhering cells, were present between the lower surfaces of the cells (Fig. 49). Many of the cells had a small number of 1 to 2  $\mu$ m diameter vacuoles (Fig. 47); in a few cells these vacuoles were much larger but since they were present in the cells prior to infection with T. vaginalis they were probably a consequence of in vitro culture. However, following infection with T. vaginalis there was generally an increase in the number of these vacuoles in cells with adhering parasites.

#### 3.3.5 General characteristics of the lesions in T. vaginalis-infected cell cultures

Figures 50 to 54 illustrate the spatial relationships between T. vaginalis and the epithelial cell monolayer in lesions present 6 h post-infection. Figure 50 is a horizontal section, taken parallel to the substratum, through the edge of a lesion and shows numerous closely-packed organisms palisaded against epithelial cells at the lesion's edge; some of the parasites have an amoeboid shape.

Vertical sections through the edges of the lesions showed that T. vaginalis frequently migrated a considerable distance under the complete parts of the monolayer at the edge of the lesions (Figs. 51 and 52). This finding confirmed the earlier light microscopic observations

(see Fig. 6) in regard to the ability of T. vaginalis to migrate under cells at the edges of lesions. This migratory activity of T. vaginalis appears to be an important factor in detaching the cells from their substratum (Figs. 52 and 53). As Figures 51, 53 and 54 show, when the epithelial cells were lifted from their substratum they became thicker and more rounded and the number of microvilli on their surfaces increased as compared to their counterparts in the intact areas of the monolayer (Figs. 47 to 49).

When the trichomonads were palisaded against the cells or lying under the monolayer their flagella and undulating membranes (UM) were restricted to the small spaces between the organisms (Figs. 50 and 55); in these situations it seems unlikely that the flagella and UM could operate normally to cause the translocation of the organisms thus the motility of T. vaginalis in the lesions must have been largely amoeboid. This possibility was supported by the finding of numerous pseudopodia in those T. vaginalis which lay beneath the monolayer (Figs. 55 and 56). Of significance, with respect to possible pathogenic mechanisms of T. vaginalis, was the discovery that the presence of many such pseudopodia caused indentations of the cytoplasm of the epithelial cells and further, that in some instances the pseudopodia lay within 250 nm of the cell nucleus (Fig. 56).

### 3.3.6 Contacts between T. vaginalis and epithelial cells

It was pointed out earlier that T. vaginalis readily adhered to both the epithelial cells and so the points of contact between T. vaginalis and the cells were examined in detail in order to understand how the parasite may form adhesions with the epithelial cell surface. Broadly, two types of contact could be distinguished; "type 1" contacts involved little differentiation of the cytoplasm of T. vaginalis at the points of contact, whereas "type 2" contacts occurred between the highly morphologically differentiated pseudopodia of the parasite and the epithelial cell surface.



Figures 57 and 58 show examples of type 1 contacts which are regions where the plasma membranes of the parasite and cell lie in parallel apposition for distances of 3  $\mu\text{m}$  or more, separated by a space of 100 nm or less. In some of these contacts there were only small points where the gap narrowed to about 10 - 20 nm (Fig. 57); in others the parasite and cell was separated by a gap of about 20 nm over the whole length of the contact (Figs. 58 and 73). The cytoplasm of the trichomonads at the contacts appeared no different in structure or content from that elsewhere in the organism; however numerous vesicles 0.1 to 0.5  $\mu\text{m}$  in diameter were commonly found close to the contacts (Figs. 57 and 58). These vesicles were similar in size to those associated with the Golgi body and may have been transporting digestive enzymes to be released at the points of contact.

Type 2 contacts are shown in Figures 59 to 64. These contacts varied in complexity from the close apposition of pseudopodia with a cell (Fig. 59) to the highly convoluted interdigitation of pseudopodia with similarly-shaped processes from the cell surface (Figs. 60, 61 and 62). Despite the difference in complexity, these contacts shared a fairly uniform 10 to 15 nm separation of the apposed membranes over much of the length of the contacts. In no case was the gap smaller than 5 nm and not once did I find conclusive evidence for the fusion of the plasma membrane of T. vaginalis with that of the epithelial cells (Fig. 62). In many of the type 2 contacts those pseudopodia of T. vaginalis intimately surrounding finger-like processes from the epithelial cells appeared to be pinching off these parts of the cell cytoplasm (Fig. 59). Sometimes there were 10 or more thin cell processes surrounded by T. vaginalis; Figures 60 and 61 show transverse and longitudinal sections, respectively, through such contacts.

A common feature of all the pseudopodia seen in type 2 contacts was the presence of an "ectoplasmic" layer at their periphery which extended for up to 2  $\mu\text{m}$  into the body of the trichomonads and excluded

almost all of the cytoplasmic constituents; this layer was composed of a matrix of microfilamentous material. In this respect the pseudopodia were morphologically similar to those seen at the points of contact both between individual T. vaginalis (Fig. 42) and between T. vaginalis and the substratum (Figs. 45 and 46). The matrix consisted mainly of short filaments 5 to 10 nm in diameter and about 50 nm in length but within this matrix some longer filaments were often aligned into bundles (Figs. 60 and 63), which sometimes pointed towards the tips of the pseudopodia. Similar filaments were found within the cytoplasm of the epithelial cells (Figs. 49 and 62); the microfilaments of epithelial cells are known to be composed of F-actin (Dipasquale, 1975) so, at least on the grounds of purely morphological similarities, the microfilaments of T. vaginalis may also be composed of F-actin but this possibility was not examined further in this study.

The difference between the types 1 and 2 contacts seen between T. vaginalis and the epithelial cells may have been largely temporal rather than absolute inasmuch as type 1 contacts may form as the trichomonads first encounter and adhere to the cells and then as the parasites become amoeboid, developing pseudopodia, and move into close apposition with the cells, type 2 contacts develop. The latter contacts probably are, as a result of the interdigitation, mechanically stronger than the former, thus enabling the parasite to resist detachment more easily; additionally the type 2 contacts involve a much larger surface area of the cell against which mechanical and chemical cytopathogenic factors from the organism can be directed.

### 3.3.7 Ruthenium red stained T. vaginalis-infected cell cultures

Some of the infected cell cultures were stained with ruthenium red (RR) in order to investigate some of the cell surface components of T. vaginalis that may be involved in the adhesions with the epithelial cells. RR binds to acidic polysaccharides and glycoproteins, including



those containing sialic acid, and its small molecular size (1.1 nm diameter) enables it to penetrate into most adhesive junctions between biological surfaces except those involving molecular contact of the surfaces, e.g. tight junctions (Luft, 1971).

Figures 65 and 66 show two T. vaginalis stained with RR. Most of the external surface of the plasma membranes of the parasite, and of the cells, is covered with a fairly uniform electron-dense deposit of RR-positive material varying from 10 to 50 nm in thickness; the staining is also present in the small spaces of the type 1 contacts between parasites and cells. Figures 67 and 68 show high magnification views of contacts between T. vaginalis and epithelial cells; RR-positive material covers the surfaces of both parasites and cells and is present between the points of closest approach of the apposed plasma membranes. In no case did I find that RR was unable to penetrate into and stain surfaces involved in either type 1 or type 2 contacts; this suggests that T. vaginalis did not form tight junctions with the cells since such junctions are impenetrable to RR (Luft, 1971). RR did not penetrate the cytoplasm of T. vaginalis but was able to enter cytoplasmic organelles which had an opening to the outside medium such as phagosomes or pinocytosomes which were in the process of formation (Fig. 67). These results showed that there was some kind of acidic component of the plasma membrane of T. vaginalis in the cell culture.

In order to ascertain whether the component(s) were an integral part of the surface of T. vaginalis and not adsorbed from the culture medium or from lysed cells, cultures of T. vaginalis were washed thoroughly in serum-free salts solution and then stained with RR. As a further test, some of these washed T. vaginalis were also incubated in neuraminidase, which removes the sialic acid residues from glycoproteins, in order to see if the RR-positive material could be removed by the enzyme. Figure 69 shows the surface of T. vaginalis which was

fixed directly (i.e. without washing) in serum-containing medium; an irregular 40 nm thick RR-positive layer is present. In contrast Figure 70 shows that the staining is almost totally removed by washing T. vaginalis in serum-free medium. Neuraminidase treatment made no difference to the amount of staining of washed trichomonads (Fig. 71). Thus it appears that most, if not all, of the RR-positive material at the surface of T. vaginalis was probably adsorbed from the culture medium and was not an integral part of the plasma membrane. Consequently there can be little intrinsic acidic material in the membrane of T. vaginalis that is able to bind RR.

### 3.3.8 Pathological changes in epithelial cells

#### 3.3.8.1 Introduction

Within each lesion in the epithelial cell monolayers there was a spectrum of pathological change which varied from completely normal cells (Figs. 50, 51 and 54), through slightly damaged cells (Figs. 65, 72 and 73) to lysed cells (Figs. 75, 76 and 77). The presence of pathological changes within a cell were almost invariably associated with the adherence of one or more T. vaginalis to the external surface of that cell. No trichomonads were found inside the cytoplasm of normal, undamaged cells in any of the many cultures examined; thus the injurious effects of T. vaginalis were probably mainly, if not entirely, exerted on the external surfaces of the cells and not intracellularly.

No pathological changes could be found in those cells lying in the intact areas of the monolayer between the lesions (Fig. 85).

#### 3.3.8.2 Early pathological changes

One of the first signs of injury to a cell was the presence of abnormally-shaped mitochondria with reduced cristae (Figs. 72 and 74). Figure 72 shows an abnormally shaped cell which projects 30  $\mu$ m into the medium; two trichomonads are lying above the cell which contains many small vacuoles, about 1  $\mu$ m in diameter, and abnormal mitochondria



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(cf. Fig. 49). Changes were noted in cells with which T. vaginalis had formed both type 1 (Fig. 73) and type 2 (Fig. 74) contacts. Other signs of injury to the cells included a rarefaction of the cytoplasm (Figs. 65 and 68) which was due to the aggregation of cytoplasmic structures into small amorphous foci leaving electron-lucent areas between them. As far as one could see most of these early changes took place without any damage to the plasma membrane of the affected cells (Figs. 73 and 74). Once the membrane was ruptured the cells lysed and their appearance changed dramatically.

### 3.3.8.3 Lysis of cells

Lysed cells were often seen lying above the monolayer where they were pushed by the burrowing activity of the trichomonads (Fig. 75). By lifting the cells off the substratum the organisms were able to attack a larger surface area of the cell. The plasma membrane of the cells was generally only ruptured at the points of contact with T. vaginalis (Figs. 76, 77 and 78). Figure 76 shows two organisms lying beneath the epithelial cell monolayer; one of the parasites has invaded the cytoplasm of a lysed cell and is phagocytosing its contents. The upper plasma membrane of the lysed cell is still complete although it is lifted away from the cytoplasm. The nucleus of the cell is swollen and electron-lucent. A similar example of cell lysis is shown in Figure 77. As Figures 76 and 77 demonstrate, the epithelial cells were lysed singly rather than in groups and so lysed cells were usually surrounded by, and retained their adhesions to, undamaged cells. The cytoplasmic organelles of the lysed cells lost much of their normal structure once the cell membrane was lysed. Figure 79 is a high power view of the area of contact between T. vaginalis and a cell; the plasma membrane is absent only at the site of contact (Fig. 78), the endoplasmic reticulum is swollen, ribosomes are absent, and the mitochondria are swollen and lack cristae.



### 3.3.9 Phagocytosis of lysed epithelial cells by *T. vaginalis*

Following lysis of the plasma membrane, the trichomonads invaded the cytoplasm of the cells and phagocytosed the cytoplasmic contents (Figs. 76, 77 and 80). The organisms protruded large pseudopodia which surrounded the contents of the cell; these contents were then enclosed in cytoplasmic membrane-bound phagosomes which moved away from the sites of phagocytosis and were seen in all parts of the body of the organisms (Figs. 79 and 80). The pseudopodia of *T. vaginalis* were morphologically similar to those seen at the type 2 contacts and contained meshworks of microfilamentous material (Figs. 81 and 82). All parts of the surface of *T. vaginalis* were able to engage in the phagocytosis of lysed-cell debris; Figure 84 shows phagocytosis taking place at the anterior pole of the parasite, close to the anterior flagella.

Scattered about the cytoplasm of *T. vaginalis* was a population of vesicles 50 to 100 nm in diameter which probably were derived from the Golgi body (see Fig. 39) although some of them may have been pinosomes. Nevertheless it is possible that these vesicles contained digestive enzymes which were released into the phagosomes since many of the vesicles were observed to be in close contact with the phagosomes (Fig. 82). Additionally many vesicles were lying close to the adhesions of *T. vaginalis* with intact epithelial cells (see 3.3.6). When axenic cultures of *T. vaginalis* were used in acid phosphatase-localisation experiments, acid phosphatase was found in the Golgi body and its associated vesicles (not shown) and also in large vacuoles and in smaller vesicles (Fig. 83). My findings confirm the results of others who have also found acid phosphatase in the same sites in *T. vaginalis* (Brugerolle, 1971; Nielsen, 1974). Thus it appears that *T. vaginalis* possesses a type of lysosomal system similar to that in other protozoa and metazoan cells (De Duve and Wattiaux, 1966) in which primary lysosomes, which would be the acid phosphatase-containing Golgi-derived vesicles, are

exported from the Golgi body to fuse with phagosomes and so form secondary lysosomes within which the phagocytosed material is digested.

### 3.4 Morphology and motility of *T. vaginalis* when adherent to a glass substratum

#### 3.4.1 Introduction

During the studies on the infection of epithelial cell cultures with *T. vaginalis* it became clear that the parasite readily adhered to both the cells and the glass substratum and that the amoeboid movements of the organisms both on and, more importantly, under the monolayer played an important role in the enlargement of the lesions and the destruction of the cells. In order to understand more about the ways in which *T. vaginalis* is capable of performing substratum-based movements, as distinct from its motility in suspension, the morphology and motility of the organism on a plane glass substratum and the dimensions of its contacts with the glass, were examined in detail using a variety of light microscopical techniques. Details of the observation chambers are given in Section 2.5.2.1; the chamber was inverted before observation thus only those *T. vaginalis* which had attached to the coverslip were photographed.

#### 3.4.2 Morphology of *T. vaginalis*

Figure 86 shows a living population of *T. vaginalis* adhering to a glass coverslip in culture. There is a diversity of morphology within the population with about half the organisms retaining a more or less spherical or rounded shape which is characteristic of *T. vaginalis* when in suspension, and the rest being flattened and irregular in outline. When examined at higher magnification, the flattened organisms were pronouncedly amoeboid and possessed large pseudopodia up to 20  $\mu\text{m}$  long. Figure 87 illustrates two amoeboid *T. vaginalis* as seen in Nomarski differential interference optics; each has protruded a single broad flat anterior pseudopodium, which contain no large organelles, and



smaller filopodial protrusions are present at the rear of the organisms. The rounded trichomonads did not have such large pseudopodia as flattened organisms, nevertheless pseudopodial and filipodial extensions of smaller size were protruded by the former organisms (Fig. 88). All the organisms possessed at least one pseudopodial extension which could be found protruding anteriorly, laterally or posteriorly from the body (Figs. 87, 88, 89 and 90). By focussing through the thickness of the organisms it was found that pseudopodia were sometimes formed on the upper surfaces (i.e. away from the substratum) of T. vaginalis and so they were not always in contact with the substratum.

T. vaginalis can divide by binary fission whilst adherent to a substratum. Figure 91 shows two daughter trichomonads about to separate following division. Occasionally large, highly-flattened organisms (e.g. Fig. 92) were observed some of which had two or more sets of flagella indicating that they were undergoing division (Fig. 93).

There was a similar morphological variation within any population of T. vaginalis adhering to the glass coverslips to that seen in the SEM study of T. vaginalis adhering to the epithelial cell cultures (compare for example Figs. 28 and 32 with Figs. 87 and 89) indicating that the development of amoeboid forms by the organism is not necessarily related to the nature of its substratum.

Most of the trichomonads on the glass coverslips were continuously changing shape, protruding and retracting pseudopodia and filopodia and many of these changes were related to the movements of the organism on the coverslips.

### 3.4.3 Motility of T. vaginalis

The motility of T. vaginalis on the coverslips was studied by both direct observation and by time-lapse still photomicrographs. Figure 94 is a sequence of stills, taken at 10 sec. intervals, which shows an irregularly shaped amoeboid T. vaginalis which develops a more polarised form as it crawls over the substratum at a speed of about 20  $\mu\text{m}$  per

min. Figure 95 is a similar time-lapse sequence, taken over a period of 11 min., of a rounded T. vaginalis. The speeds of the rounded T. vaginalis were not greatly different from that of the more flattened organisms; however the net displacement in a given time was lower in the former since they often moved around in circular paths whereas the flattened trichomonads showed a greater persistence in the direction of their movement. In general the body of the trichomonads moved in the direction of protrusion of the largest pseudopodium (e.g. Fig. 95, frames 2 to 5) and since pseudopodia could develop or disappear within a few seconds so the direction of movement of an organism frequently changed during any one period. Whilst T. vaginalis crawls on a substratum in an amoeboid manner, its flagella and undulating membrane were invariably still beating and so may have contributed to some extent to the forces required to bring about the displacement of the organism.

By studying the movements of T. vaginalis it was found that the presence of both rounded and flattened organisms within a population of T. vaginalis adhering to glass (Fig. 86) was not a sign of any fundamental intrinsic difference between the two morphological groups but rather a reflection of the ability of T. vaginalis to change from a rounded to a flattened shape, and vice versa, in a short period of time. Figure 96 illustrates this phenomenon occurring in a group of 5 T. vaginalis. One organism remains almost stationary but the others continuously change shape as they move on the coverslip; the assumption of a flattened morphology is easily seen by the reduction in the phase halo, characteristic of rounded organisms, as the organisms spread onto the substratum. From these observations it was clear that the rounded organisms were no less adhesive than their flattened counterparts which had greater areas of their surface adherent to the substratum. Indeed if, as frequently happened during a long period of observation, a rounded trichomonad detached itself from the coverslip and sank down through the chamber to the lower coverslip it reattached itself to that substratum



and exhibited a similar cycle of flattening and rounding up. For these reasons it is possible that the presence of numerous T. vaginalis in the decanted supernatants of the epithelial cell cultures when they were fixed for light or electron microscopy (see 3.1.3, p. 68) did not mean that these unadherent, freely swimming organisms had not at some time been attached to the epithelial cell monolayer.

#### 3.4.4 Substratum contacts of T. vaginalis as seen by interference reflection microscopy

Interference reflection microscopy may be used to examine the spatial separation of the lower surface of a cell from its substratum when it is adhering to a plane glass coverslip in culture. The optical principles of interference reflection (IR) microscopy have been described in detail by Curtis (1964) and Izzard and Lochner (1976) and will not be reiterated in detail here. Briefly, the IR image is derived from the first order interference pattern generated by the light reflected from each of the boundaries of the thin film of culture medium lying between the trichomonads and the glass coverslip to which they were adhering, i.e. the glass/medium interface and the medium T. vaginalis interface. Where the organism is lying within 10 nm of the glass substratum the IR image will be black; the image will progressively lighten, through grey to white, as the organism lies further away from the glass up to a distance of about 150 nm. If the organism is further from the substratum than 150 nm no IR image is obtained.

Figures 97 to 100 demonstrates the typical IR images of T. vaginalis when adhering to a glass substratum in culture. Each organism is shown in both phase contrast and interference reflection optics. When T. vaginalis was flattened and amoeboid all of its lower surface lay between 10 and 30 nm from the substratum as was shown by a dark grey IR image (Figs. 97 and 99). The rounded organisms (Figs. 98 and 99) were generally in less close contact and their IR images were light grey with patches of white showing that they were less closely adherent to the

glass than the flattened organisms; the white areas demonstrated that a separation of about 100 nm exists between some parts of the ventral surface of the organism and the glass. Those organisms which were almost completely rounded up as shown by their phase contrast haloes (e.g. the organism on the right in Fig. 99) had only a very small part of their surface lying less than 100 nm from the substratum.

Typically the pseudopodia of T. vaginalis were always among those parts of the body in closest apposition to the substratum (Figs. 98, 99 and 100) although in long pseudopodia only the leading edge lay 10 to 30 nm from the glass with the rest further away. Figure 100 shows a pseudopodium of which only the tip is seen in IR; the proximal parts of the pseudopodium are not seen in IR and so lie about 150 nm or more above the substratum.

#### 3.4.5 Summary: on the amoeboid motility of a flagellate

It is axiomatic that if an organism is to perform active substratum-based movements then it must make temporary but stable attachments to its substratum in order that tractive forces, developed by the organism, may be applied to the substratum and so cause its forward movement over the substratum enabling it to form new adhesions with fresh parts of the substratum. How then can a protozoan like T. vaginalis which is equipped with four anterior flagella and an undulating membrane for swimming in a liquid medium fulfil these criteria for substratum-based movements? The combined light and electron microscopical studies presented here provide some of the answers to this problem.

Firstly the surface of T. vaginalis is sticky enabling it to adhere to both a glass coverslip and to the surfaces of epithelial cells. These adhesions were clearly of sufficient strength and stability to allow T. vaginalis to remain attached to an inverted coverslip and also to resist detachment from the cell cultures when they were shaken gently. Secondly T. vaginalis can form pseudopodial and filopodial processes which it extends away from its body and over the surrounding substratum



and these processes can make close, 10 to 30 nm, contacts with the substratum. Since the movement of the amoeboid organisms generally proceeds in the direction of protrusion of the largest pseudopodium it is probable that the tractive forces necessary for displacement are largely developed within the pseudopodia and exerted between the substratum adhesions of the pseudopodia and the central parts of the body. The presence of microfilaments, to the virtual exclusion of all other organelles and structures, within the pseudopodia suggests that they play a part in the mechanism of protrusion of the pseudopodia and of movement of the body as a whole.

### 3.5 Enzyme assays on *T. vaginalis*

#### 3.5.1 Preparation of homogenates of *T. vaginalis*

The techniques used to prepare homogenates and lysates of pellets of living *T. vaginalis* (see 2.7.2.1) were designed to obtain two fractions of the organisms that could be assayed for their enzyme content, the cytosol fraction and the particle fraction. The cytosol fraction contained the soluble components of the cytoplasm of *T. vaginalis* from which all insoluble or particulate material had been removed by centrifugation at 105,000 g; thus any enzymes present in this fraction would be those which were not bound to any of the organelles or structures present within the cytoplasm of *T. vaginalis*. In contrast, the particle fraction contained those enzymes that were bound to the insoluble components of the cytoplasm or contained within membrane-bound particles and organelles such as phagosomes, vesicles, endoplasmic reticulum and the Golgi body. The presence of an enzyme within a membrane-bound particle frequently means that this enzyme is not accessible to the exogenous substrate of that enzyme supplied in the assay procedure since the membrane acts as a permeability barrier to the substrate (Muller, 1973). For this reason, the particle fractions of *T. vaginalis* were sometimes also prepared in the presence of the

non-ionic detergent Triton X-100 which solubilises membranes thus allowing access of the substrate to the enzyme.

### 3.5.2 Acid phosphatase

The results of assays for acid phosphatase on two stocks of T. vaginalis are shown in Table 4. In this assay only, the homogenates were treated with a preliminary low speed centrifugation (500 g) which removed unbroken cells, nuclei and large cytoplasmic structures such as the axostyle, flagella and mastigont apparatus. The particle fraction was examined as a negatively stained preparation in the electron microscope and was found to be comprised mainly of membrane-bound organelles such as hydrogenosomes, vesicles, phagosomes and elements of the endoplasmic reticulum and Golgi body.

The results show that the highest specific activity of acid phosphatase in T. vaginalis is present in the particle fraction. For LUMPs 889 and 993 the total specific activity of the particle fraction was not dissimilar, however the ratios of the activities of the particle and cytosol fractions differed by an order of magnitude, being 5.5:1 and 55:1 respectively. This difference may of course have been real or alternatively artefactual and due perhaps to the leakage of enzyme from the particles of the organisms of LUMP 889 during preparation as a result of insufficient osmotic protection. Nevertheless the results of these assays when added to the results of the cytochemical-localisation studies described earlier (see 3.3.9) demonstrate that T. vaginalis contains the enzyme acid phosphatase and that most of the activity of the enzyme is associated with particulate membrane-bound structures in the cytoplasm of the organism such as the Golgi body, phagosomes and small vesicles.

### 3.5.3 $\beta$ -glucuronidase

T. vaginalis from LUMP 849 was assayed for  $\beta$ -glucuronidase activity. A homogenate of  $10^8$  organisms comprising of both the cytosol fraction and the solubilised particle fraction was assayed,



however no enzyme activity could be demonstrated under the assay conditions. Therefore it is possible that either T. vaginalis contains no  $\beta$ -glucuronidase activity or else the enzyme is present in such small amounts that cannot be detected by a standard assay technique.

#### 3.5.4 Neuraminidase

The results of neuraminidase assays on two stocks of T. vaginalis are shown in Table 5. No enzyme activity was demonstrated by either the living organisms or the lysates of T. vaginalis from LUMPs 840 and 896, as measured by their ability to release sialic acid from sialomucoids. The slightly higher activity of the cytosol and solubilised particles of LUMP 896 over the H<sub>2</sub>O controls, i.e. 3.44% compared to 2.6%, is most likely a sign of experimental error and cannot be relied on as indicative of neuraminidase activity without further investigation.

#### 4. DISCUSSION

##### 4.1 The *T. vaginalis*-infected RK 13 epithelial cell culture as a model of human urogenital trichomoniasis

Although studies on the interaction of *T. vaginalis* with the vaginal and cervical epithelia of females with trichomonal vaginitis (Koss and Wolinska, 1959; Nielsen and Nielsen, 1975; Garcia-Tamayo et al., 1978) have been of great value in our understanding of the mechanisms of pathogenicity of the parasite we always have to bear in mind the possibility that the behaviour and pathogenicity of the parasite may be modified, either directly or indirectly, by variations in the local or systemic conditions of its host. For example, it is well known that exacerbation of the symptoms of trichomonal vaginitis may occur during the menses (De Leon, 1971) and during pregnancy, (Schofield, 1972). For these and other reasons it is, I believe, important that we have a controllable experimental system in which the inherent mechanisms of pathogenicity of *T. vaginalis* may be analysed in detail and where factors such as innate resistance, hormonal status and concomitant bacterial flora, which are possible complicating factors present in natural infections (see review of Honigberg, 1978b), are absent. In this thesis I have shown that the *T. vaginalis*-infected RK 13 epithelial cell culture can offer a suitable experimental method of elucidating the mechanism of pathogenicity of *T. vaginalis*; the following points concerning the design of these experiments merit some discussion with reference to earlier studies of *T. vaginalis* in cell cultures.

##### 4.1.1 Choice of cell culture

Rabbit kidney tubule (RK 13) epithelial cells were selected because they are easily cultured in vitro and form a coherent monolayer of flattened polygonal cells on a glass substratum. The monolayers were therefore morphologically similar to the outer layer of squamous epithelial cells of the human vagina and ectocervix and since the



latter cells are not commercially available as a continuous cell line, the RK 13 cells are a suitable alternative. In fact, earlier studies using a wide range of avian and mammalian tissue cells (listed in section 1.16.1) suggest that T. vaginalis behaves similarly to different cell types however since only epithelial cells, of whatever origin, form a cohesive monolayer of cells in culture (unlike, say, fibroblasts which are spindle-shaped so that even in dense cultures there are frequently small spaces between adjacent cells) they are to be preferred as a model of the vaginal and cervical epithelium.

#### 4.1.2 Choice of T. vaginalis

The results of the IM, SEM and TEM studies of T. vaginalis-infected RK 13 cell cultures which I have presented in this thesis represent the collected findings of separate studies using four stocks of T. vaginalis, LUMPs 866, 873, 889 and 896. Under the experimental conditions used in this study, I found no significant qualitative differences in the behaviour of the four stocks in the cell cultures. Each stock destroyed a monolayer of cells within about 36 h and the sequence of events leading to the death of the cell cultures as a result of infection with each stock appeared to be similar. It is of course possible that there were some differences in the behaviour and mechanisms of cytopathogenicity of each stock which I did not detect, however I believe that they must have been small and they in no way invalidate the main findings of this study.

#### 4.1.3 Selection of size of inoculum

One of the important considerations in any study of T. vaginalis-infected cell cultures is the selection of a suitable number of parasites for inoculation of the cell cultures so that the most informative picture of the cytopathogenic changes associated with the interaction of T. vaginalis and the cells may be obtained. In this study I used an inoculum of  $2 \times 10^5$  parasites per RK 13 cell culture chiefly for two

reasons. With this inoculum of parasites large lesions, ideal for a close study at both the light and electron microscope level of the T. vaginalis - RK 13 cell interaction, occurred within 6 to 10 h post inoculation at a time when the conditions in the culture medium, such as the pH and the level of nutrients, had not altered significantly from those prevailing at the start of the infection and so were unlikely to be a contributing factor in the cytopathogenicity of T. vaginalis. Furthermore other workers (Kulda, 1967; Farris and Honigberg, 1970) employed similar numbers of parasites in their studies of T. vaginalis-infected cell cultures (see Table 2) so the results of this study may be more directly compared to these earlier studies. On the other hand Christian et al. (1963) infected HeLa cell culture with only  $5 \times 10^3$  T. vaginalis but despite this small inoculum of parasites obtained a picture of the sequence of events leading to the destruction of the cells by the parasites which bears many similarities to this study; however I did not examine the possibility that low parasite inocula of the stocks of T. vaginalis at my disposal were capable of producing the same cytopathogenic effects on RK 13 cells as the relatively high inoculum of  $2 \times 10^5$  which I routinely used to infect the cell cultures.

Inasmuch as many workers, including myself, have infected cell cultures with sufficient numbers of T. vaginalis to cause extensive cytopathological changes in the cells thereby adding to our understanding of the mechanism of pathogenicity of T. vaginalis in natural infections in humans, it is apparent that future studies in which cell cultures are infected with very low numbers of parasites, numbers insufficient to cause much cell injury, may prove of equal value in elucidating the nature of latent asymptomatic infections in humans. This possibility is suggested by the work of Christian et al. (1963) following their demonstration that HeLa cell cultures inoculated with only 500 T. vaginalis could be maintained for up to 1 month with minimal cell injury and little parasite growth, a situation which, they argued,



paralleled latent trichomonal infections in humans in which the patient is an asymptomatic, but potentially infective, host.

#### 4.2 Pathogenicity of *T. vaginalis* in RK 13 epithelial cell cultures

##### 4.2.1 General characteristics

The results of this study indicate that, under the experimental conditions employed, the four stocks of *T. vaginalis* selected for study are highly pathogenic when inoculated into cultures of monolayers of RK 13 epithelial cells and the parasites are capable of totally destroying the monolayers within about 36 h. The most significant feature of the infections was that during the first few hours post-inoculation of the cultures with parasites cytopathological changes and cell lysis occurred not throughout the monolayers but in discrete foci, i.e. the lesions. The lesions enlarged as the infection progressed as a result of the parasites' attack on and lysis of a narrow band of cells, not more than 3 cells wide, which lined the edges of the lesions and against which the parasites were palisaded. Having destroyed this band of cells, and thereby enlarged the lesion, the parasites continued to move outwards attacking the as yet undamaged and intact areas of the monolayer. This feature of the *T. vaginalis*-RK 13 monolayer interaction is one which others have also observed in studies using different types of cell culture (Christian et al., 1963; Kulda, 1967; Farris and Honigberg, 1970) but, unlike this study, these authors did not, or were unable to, examine in any detail the fine structural aspects of the interaction of *T. vaginalis* and the cells at the edges of the lesions.

This study enables us to understand the lesion-forming ability of *T. vaginalis* in cell cultures more comprehensively than previously; the reasons for this ability are, I believe, three fold: a) the tendency of *T. vaginalis* to aggregate into clumps of up to 200 individuals soon after inoculation into the cell cultures, b) the adhesion of these aggregates to the monolayer of cells, and c) having cleared a small area

of the monolayer by lysis of the cells beneath the aggregates, the ability of the organisms to adhere to the glass substratum, develop pseudopodia and then move in an amoeboid manner both under and over the cells at the edge of the lesion destroying them as the parasites move outwards.

Since it has been observed previously (vide supra) and may therefore be a characteristic of many stocks of T. vaginalis, the lesion-forming ability of the organism suggests a possible method of an in-vitro assay of the cytopathogenicity of different stocks of T. vaginalis. By using a standardised monolayer of cultured cells inoculated with a standard number of parasites of each stock of T. vaginalis, the areas of the lesions relative to total area of the monolayer after, say, 6 h of infection could be used as a measure of the inherent pathogenicity of the stocks and so provide a suitable, and certainly less expensive, alternative to Honigberg's (1961) SC mouse assay which is at present the main method of assaying the inherent pathogenicity of T. vaginalis.

#### 4.2.2 Pathological changes in epithelial cells

T. vaginalis disturbed the integrity of the RK 13 monolayers in two ways, firstly by detaching individual cells from the coverslips or from neighbouring cells and secondly by causing degenerative changes within the cells which lead to lysis.

The activities of the amoeboid trichomonads at the edges of the lesions caused some of the cells to adopt a more rounded morphology as a result of the retraction of the peripheral areas of their cytoplasm, while other cells which the organisms almost totally detached from the substratum, or from neighbouring cells, assumed a nearly spherical shape. The ability of T. vaginalis to detach cells from a surface, whether it be living or non-living, is something which Christian et al. (1963) also noticed in T. vaginalis-infected HeLa cell cultures; they saw parasites "tumbling" detached HeLa cells with their flagella in the



centre of lesions in the HeLa cell monolayer.

The pathogenic effects of T. vaginalis on the RK 13 cells caused fine structural changes within the cells which included the swelling of mitochondria and a loss of their cristae, an increase in the numbers of cytoplasmic vacuoles, a disorganisation and rarefaction of the cytoplasmic matrix and an increase in autocytophagocytosis. These changes are broadly similar to those seen in the vaginal and ectocervical epithelia in acute human trichomonal vaginitis (De Leon, 1971; Honigberg, 1978b). At 6 h post-inoculation of the RK 13 cultures with T. vaginalis these changes were only present in cells to which parasites were adhering which suggests that contact between T. vaginalis and a cell surface plays an important part in cytopathogenesis. Inasmuch as these changes occurred in cells which possessed an intact plasma membrane it seems likely that they were caused by the actions of the parasites on the surfaces of the cells. The changes may have been a result of the breakdown of the selective permeability of the plasma membrane which in turn may have caused a loss of soluble cytoplasmic components, a reduction in the uptake of nutrients, an accumulation of toxic metabolic waste products and an increase in hydrostatic pressure of the cytoplasm due to a loss of the osmotic control of the plasma membrane over cytoplasmic tonicity. Unfortunately these rather perfunctory speculations as to the consequences of the attack of T. vaginalis on the plasma membrane of RK 13 cells are not ones which are easily validated simply on the basis of a fine structural study as this present study and so further investigation of the physiological changes in cells attacked by T. vaginalis is required.

#### 4.2.2.1 The question of intracellular T. vaginalis

It has been suggested previously that T. vaginalis can cause injury not only from its actions on the surfaces of cells but also from within a unit-membrane bound vacuole inside a healthy cell. In an EM

study of T. vaginalis-infected mouse livers Brugerolle et al. (1974) noted that the parasites were frequently phagocytosed by macrophages and that, enclosed within a phagosome in the cytoplasm of the macrophages, the trichomonads caused vacuolation, dilatation of endoplasmic reticulum, and plasma membrane breakage in these cells. Similarly Farris and Honigberg (1970) in a IM study of T. vaginalis-infected chick liver cell cultures, comprising macrophages, fibroblasts and epithelial cells, found that the macrophages ingested the parasites and were frequently destroyed by their ingested trichomonads. However evidence that T. vaginalis can invade healthy non-phagocytic tissue cells is less conclusive. Frost et al. (1961) reported a single instance when they observed a trichomonad in a vaginal squamous epithelial cell in a smear from a woman with trichomonal vaginitis, and Farris and Honigberg (1970), in the same study as above, observed chick fibroblasts and epithelial cells which had one, and sometimes two, parasites lodged within "vacuoles" in their cytoplasm. In both these reports the evidence that the trichomonads were intracellular was based on light microscopic examination of fixed and stained material and so it is quite possible that rather than being within the cells, the trichomonads were in fact lying under the cells giving a false impression of intracellularity.

In this study I have shown that T. vaginalis commonly crawled under healthy epithelial cells but did not invade them whereas the parasites did invade cells in which the plasma membrane had been ruptured and which showed extensive structural degeneration. Although many of the undamaged RK 13 cells contained large vacuoles (e.g. Fig. 2) I did not find parasites within them and so I would agree with Hogue (1947) who also noted that chick embryo epithelial cells, 20 to 24 h post-infection with T. vaginalis, contained many vacuoles which "although .... much larger than the trichomonads they were never seen



to contain any of the animals". Since the majority of the chick fibroblasts and epithelial cells seen by Farris and Honigberg (1970) to be harbouring T. vaginalis were swollen and had a highly vacuolated cytoplasm these authors did however consider the possibility that their parasites entered the cultured cells after the process of degeneration had set in, but they argued that since they found parasites during the first few hours of the infection within what they considered healthy cells this possibility was unlikely; but equally, it may be argued that what constitutes a "healthy" cell as seen at low resolution under the light microscope by Farris and Honigberg may be one which at the EM level is showing signs of degeneration so it is a pity that these workers did not back up their assertions with electron micrographs. The question of intracellular T. vaginalis in healthy non-phagocytic cells is one which could be resolved in the future with the aid of a combined cinemicrographic and electron microscopic study of parasite-cell interactions.

#### 4.2.3 Specificity of T. vaginalis-induced cytopathogenesis

The pathological changes which T. vaginalis caused in the RK 13 cells are typical of T. vaginalis infections in general whether they be in cell cultures (Farris and Honigberg, 1970), in laboratory mice (Brugerolle et al., 1974) or in the human vagina (De Leon, 1971). However these changes are not specific to T. vaginalis since qualitatively similar pathological changes are found in chick liver cell cultures infected with pathogenic strains of other trichomonads such as Tri. foetus (Kulda and Honigberg, 1969) and T. gallinae (Honigberg et al., 1964b). The mitochondrial distension, cytoplasmic vacuolisation and rarefaction which my T. vaginalis produced in RK 13 cells are also changes which Entamoeba histolytica (Evans strain) produces in the same cells (Knight, Bird and McCaul, 1975) and which occur within the caecal tissue of turkeys and chickens infected with Histomonas meleagridis (Lee, Long, Millard and Bradley, 1969) and Tet. gallinarum (Lee, 1972) respectively.

The ability of T. vaginalis to form lesions in cell monolayers is a characteristic which is also shared by E. histolytica (Knight et al., 1975), and Naegleria fowleri and Acanthamoeba culbertsoni (Cursons and Brown, 1978). Furthermore, these changes may also be produced in cell cultures by non-living agents such as certain drugs, poisons and by irradiation (Bang, 1966).

#### 4.2.4 Roles of the adhesiveness and amoeboid motility of T. vaginalis in cytopathogenicity

In this study I have indicated that the adhesiveness and amoeboid motility of T. vaginalis may play important roles in the ability of the parasite to injure cells. Before considering the mechanisms of pathogenicity of T. vaginalis, I shall consider in some detail these two important, but hitherto little-studied aspects of the biology of T. vaginalis.

### 4.3 Adhesiveness of T. vaginalis

#### 4.3.1 Specificity

In this study I have shown that T. vaginalis is able to adhere to other trichomonads, to RK 13 epithelial cells and to glass coverslips. Other workers have shown that the organism will adhere to a wide variety of cells, both in vitro and in vivo, including avian and mammalian fibroblasts and epithelial cells (Hogue, 1943; Christian et al., 1963; Kulda, 1967; Farris and Honigberg, 1970) and human vaginal squamous epithelial cells (Nielsen and Nielsen, 1975). It has also been known for some time that T. vaginalis readily adheres to non-living substrata such as glass (Lumsden et al., 1966), tissue-culture grade plastic (Cappuccinelli, Lattes and Cagliani, 1973), and to fibres of unknown constitution which occur in culture media, although not if they are of vegetable origin such as cotton fibres (Lumsden et al., 1966). So it is evident that the adhesiveness of T. vaginalis is largely non-specific and that it can adhere to a wide range of both organic and inorganic



substrata. This non-specificity of adhesiveness is therefore a feature which T. vaginalis shares with some other protozoa, such as the rhizopod amoebae (Jeon, 1973) and kinetoplastid flagellates (Vickerman, 1972), and most metazoan cells, all of which will also adhere to a variety of substrata.

#### 4.3.2 Areas of the surface of T. vaginalis involved in adhesion

I found that in general T. vaginalis adhered to the epithelial cells and the glass coverslips with its body and pseudopodia. Except for the single instance when the flagella were wrapped around a cell process (Fig. 31), I could not show that the flagella and undulating membrane of T. vaginalis were involved in adhesion. This is perhaps surprising in view of the fact that amongst the kinetoplastid flagellates adhesion of the flagella to a variety of substrata such as cellulose, chitin and membranous debris (Brooker, 1970, 1971; Vickerman, 1972) is a common occurrence. It is possible that the plasma membrane of the flagella and undulating membrane of T. vaginalis is less sticky than that over the rest of the body however such a possibility does not explain the fact that the recurrent flagellum adheres to the undulating membrane over the whole of its length. The answer to this puzzle may be quite simply that the continual motion of these organelles means that they do not stay long enough in contact with a surface to be able to adhere to it. In contrast the immobile axostylar projection of T. vaginalis was sometimes involved in the adhesion of the organisms to the epithelial cells but only during the first phase of the infection as the parasites settled onto the cells. Both Hogue (1943) and Christian et al. (1963) also noted that T. vaginalis adhered via its axostyle to the surfaces of cells.

#### 4.3.3 Morphology of adhesions between T. vaginalis and surfaces

Most of our understanding of the morphology and function of adhesive junctions between cells has come from studies on metazoan cells but it

is becoming clear that some of the characteristics of metazoan adhesions are shared by protozoans (Vickerman, 1972). Broadly, four types of adhesive junctions may be distinguished, on both morphological and physiological grounds, in metazoan tissues (Trinkaus, 1969; McNutt and Weinstein, 1973): a) tight junctions, in which the apposed plasma membranes are in molecular contact, which function as a permeability barrier in tissues; b) gap junctions, in which the apposed membranes are separated by a gap of about 2 to 6 nm and which are involved in electrotonic coupling and intercellular transport between cells; c) desmosomes, which are characterised by a gap of about 10 nm and the presence of dense cytoplasmic plaques, associated with 5 to 10 nm diameter tonofilaments, subjacent to the apposed membranes; these junctions are largely responsible for the structural integrity of tissues; and finally, d) intermediate junctions, which are regions of 10 to 20 nm separation of the apposed membranes which show little specialisation of the subjacent cytoplasm but are one of the most frequently encountered types of intercellular adhesions, and are also typical of the adhesions of cells to non-living substrata. Using this classification can we relate the adhesions of T. vaginalis to those of metazoan cells?

Firstly I did not find any tight junctions between T. vaginalis and the epithelial cells which lends some support to Vickerman's (1972) statement that tight junctions do not occur at protozoan-host cell contacts. Similarly I found no clear evidence that gap junctions occur at the areas of contact of T. vaginalis and cells; on only a very few occasions did the plasma membrane of the parasite approach to about 5 nm from that of the cells. On the other hand Nielsen and Nielsen (1975) have shown that in the areas of close contact between adjacent T. vaginalis, and between T. vaginalis and human vaginal epithelial cells contacts of the size of gap junctions do occur, but whether such junctions between trichomonads are sites of interparasite communication is



an open question.

No desmosomal-like junctions were found between T. vaginalis and either the epithelial cells or the glass coverslip. In this respect T. vaginalis would appear to differ from the kinetoplastid flagellates, for example, which form hemidesmosomal plaques at the sites of attachment of their flagella to non-living substrata (Brooker, 1970, 1971) and full desmosomal plaques at the adhesions of the flagellum to their bodies (Vickerman, 1972) but, as Vickerman and Preston (1976) record, there has been only one report of these flagellates forming desmosomes with the plasma membrane of their host cells.

Turning now to intermediate junctions, it is clear from this study that the majority of the sites of adhesion between T. vaginalis and the epithelial cells were typified by a gap of 10 to 20 nm so on morphological grounds the adhesions of T. vaginalis to cells may be called intermediate junctions. This type of junction has been found previously between T. vaginalis and other types of cells including human vaginal squamous epithelial cells (Tamayo et al., 1972; Nielsen and Nielsen, 1975; Ovccinikov et al., 1975; Garcia-Tamayo et al., 1978) and mouse hepatocytes (Brugerolle et al., 1974); furthermore, intermediate-type junctions are seen between the tissue-invasive forms of H. meleagridis (Lee et al., 1969) and Tet. gallinarum (Lee, 1972) and their host cells, and between E. histolytica and cultured RK 13 epithelial cells (Knight, et al., 1975), consequently the ability to form intermediate junctions with host cells may be a property of many protozoa.

The 10 to 20 nm-gap or intermediate junction was also present at the regions of adhesion between trichomonads; however in this situation a second type of junction was occasionally present in which the plasma membranes of the adhering organisms were separated by a gap of 75 nm (Figs. 43 and 44). This type of junction has not been previously described in T. vaginalis. One interesting feature of these junctions is that, unlike the intermediate junctions between trichomonads, the

apposed membranes were linked by extracellular fibrils, thus the junction resembled a desmosome except that no cytoplasmic plaques or filaments were found, but at present the nature of these fibrils and the functional significance of the junction remains a mystery.

Finally, interference reflection microscopy (IRM) studies have shown that the adhesions of various metazoan cells, such as chick fibroblasts (Abercrombie and Dunn, 1975) and rabbit leucocytes (Armstrong and Lackie, 1975), to plane glass coverslips are of the intermediate junction type, in that the gap between the cell surface and glass coverslip is about 10 to 30 nm. In this study I have shown that when T. vaginalis adheres to glass much of its lower surface lies within 30 nm of the substratum; this demonstrates a further similarity of the adhesive junctions of T. vaginalis with those of metazoan cells.

This is the first study in which IRM has been used to investigate the adhesions of T. vaginalis to glass. The technique of IRM, although widely used to study metazoan cells, has been little used in protozoology except by Opas (1978) and Preston and King (1978). In an IRM study of the adhesions to glass of Naegleria gruberi, a free-living amoebophilic flagellate, Preston and King (1978) showed that, like my findings on T. vaginalis, the points of contact of amoeba to glass were characterised by a gap of about 20 nm; however unlike T. vaginalis, which I have shown may have large areas of its lower surface lying 10 to 30 nm from a glass substratum, Preston and King found that N. gruberi had only a few, discrete parts of its lower surface in such close contact, the rest of the amoeba lay more than 110 nm from the glass. Amoeba proteus adheres to glass in a manner similar to N. gruberi (Opas, 1978).

#### 4.3.4 Mechanisms of adhesion in T. vaginalis

What mechanisms may be involved in the formation of the intermediate junctions between T. vaginalis and surfaces? Curtis (1967, 1972) considers that there are basically only two general mechanisms that can



account for the existence of the 10 to 20 nm gap of intermediate junctions, the "secondary minimum mechanism" is derived from the physical nature of cell surfaces, whereas the "bridging mechanism" involves the formation of a chemical bridge between the surfaces. However, as Curtis (1972) points out, there is no unequivocal experimental evidence in favour of, or against, either of these mechanisms.

The "secondary minimum" theory (Curtis, 1967) states that when two apposed cell surfaces are 10 to 20 nm apart they adhere because they are in the secondary minimum of the potential energy diagram of interaction; the minimum occurs when the attractive forces between the surfaces, due to the London-van der Waals dispersion force of each surface, are balanced by the electrostatic forces of repulsion, which in turn are due to the negative charge present on most cell surfaces. The attractive London-van der Waals forces arise from the polarisation of the charges of neutral atoms and so, at least theoretically, these forces may be present at the surfaces of T. vaginalis and of the objects with which it comes in contact. The repulsive electrostatic forces between metazoan cell surfaces are due mainly to the fact that most, if not all, metazoan cells possess sialic acids which are terminally located on the side chains of membrane glycoproteins and it is these acids which give the surface its negative charge (Lloyd, 1975). However in this study I have shown from the inability of the plasma membrane of washed organisms, treated with neuraminidase, to bind ruthenium red that T. vaginalis can have little sialic acid at its surface. Of course the apparent lack of sialic acid does not mean that there are not other non-ruthenium red-binding acidic molecules at the surface of T. vaginalis that may be involved in a secondary minimum adhesion; Rodriguez-Martinez, Rosales, Bello and Moreno (1973) found that T. vaginalis stained "adequately" with periodic-acid Schiff stain indicating acidic polysaccharides were present at the surface of the parasite, although it must be noted that their parasites were stained

directly after removal from human vaginae and so the organisms may have adsorbed acidic molecules on their surfaces from vaginal discharge. Nevertheless other studies on protozoa have shown that their surfaces are negatively charged and also that the charge is not necessarily due to sialic acids; a net negative surface charge was demonstrated on N. gruberi by electrophoretic mobility studies (Forrester, Gingell and Korchoda, 1967) and by the ability of the amoeba to bind cationised ferritin evenly all over its surface (King and Preston, 1977). Similarly Dwyer (1975) showed that the surface of blood and culture forms of Trypanosoma lewisi binds cationic dyes and that neuraminidase, which specifically removes sialic acids from glycoproteins, did not diminish the staining which indicates that the dyes were binding to types of acidic molecules other than sialic acid. Further, the free-living soil amoebae A. proteus and Acanthamoeba castellanii have no sialic acid in their plasma membranes, the major acidic group in their membranes is respectively phosphate (Allen, Ault, Winzler and Danielli, 1974) and lipophosphoglycan (Korn, Dearborn and Wright, 1974).

The bridging theory (see Curtis, 1967) states that the apposed surfaces are bridged by molecules which are bi- or polyfunctional so that one end of the bridging agent binds to one surface and the other to the opposite surface. Some evidence that a surface molecule may be involved in the adhesion of T. vaginalis to glass has come from the work of Cappuccinelli and colleagues on a strain of T. vaginalis (FC) which readily adheres to glass coverslips in culture (Cappuccinelli, Lattes and Cagliani, 1973). Cappuccinelli (1973) showed that the adhesiveness of this strain was almost totally abolished if the culture medium contained either 4 mg per ml trypsin or 0.2 mM ethylene-diamine-tetra-acetic acid (EDTA). In a later paper Cappuccinelli, Cagliani and Cavallo, (1975) showed that EDTA removes a surface concanavalin-A-binding glycoprotein from the FC strain of T. vaginalis; they were able



to isolate and purify this glycoprotein and demonstrated that it was involved in adhesion in the following elegant way.  $4 \times 10^4$  T. vaginalis were incubated in 5 mM EDTA, in phosphate buffered saline (PBS) pH 7.0, for 15 min. at 37°C, and then washed free of EDTA, placed in PBS containing 10 µg per ml. cyclo-hexamide (a protein synthesis inhibitor which prevented the organism resynthesising the removed glycoprotein) and allowed to attach to glass coverslips for 1 h. They found that following this treatment only 7 per cent of the parasites were adhering after 1 h, however this percentage increased to 63 when they added 10 µg per ml of the surface glycoprotein indicating that the glycoprotein was specifically involved in the adhesion of T. vaginalis to glass. Although Cappuccinelli's studies suggest that EDTA, and possibly trypsin also, interfere with adhesion by removing a surface glycoprotein, we cannot necessarily assume that this molecule provides part of a bridging mechanism in the adhesion of T. vaginalis since it is equally possible that the removal of the glycoprotein alters the surface charge of the parasite so reducing the opportunity of the organism to form adhesion by a secondary minimum mechanism. However these studies do imply that T. vaginalis shares glycoprotein-dependent mechanisms of adhesion with metazoan cells (Lloyd, 1975; Lloyd and Cook, 1975).

In further investigations, Cappuccinelli, Cagliani and Cavallo (1973) and Cappuccinelli and Varesio (1975), have found that colchicine and vinblastine, which are inhibitors of microtubule assembly, do not affect the adhesion of T. vaginalis, whereas cytochalasin B at concentrations in excess of 50 µg per ml causes about 50 per cent inhibition. Cytochalasin B interferes with microfilament-mediated contractile processes in cells, such as motility and phagocytosis (Tanenbaum, 1978); however in metazoan cells inhibition of such processes may be achieved with concentrations in the order of 0.5 to 10 µg per ml cytochalasin B

whereas Cappuccinelli and Varesio (1975) found that similar concentrations were without effect on the adhesion of T. vaginalis which suggests that this drug may be of little value in elucidating the mechanisms of adhesion of the parasite. On the other hand cytochalasin B may prove to be of value in studies on the microfilaments of T. vaginalis and their involvement in amoeboid movement and phagocytosis (see 4.4.2).

In summary, until we know more about the surface chemistry of T. vaginalis we can only speculate as to what mechanism are involved in the adhesion of T. vaginalis to surfaces and whether they involve an electrostatic interaction, as in the secondary minimum mechanism, or specific surface glycoproteins, as in the bridging mechanism. Among the questions still unanswered is that concerning the role of calcium in the adhesions of T. vaginalis. Calcium ions are important in metazoan cell adhesion (Trinkaus, 1969) and since EDTA is a calcium chelator, the release of a surface glycoprotein from T. vaginalis by EDTA might suggest a role for calcium in stabilising the surface of T. vaginalis, and maintaining its adhesiveness.

#### 4.3.5 Importance of adhesion in the biology of T. vaginalis

The ability of T. vaginalis to adhere to surfaces is important for several reasons. Firstly adhesion, when coupled with an internal contractile system, enables the organism to locomote in an amoeboid manner since a priori no cell can move on a substratum unless it can make stable adhesions to that substratum. Secondly the adhesiveness of the parasite may be important in enabling it to remain in a particular site in its host. In human trichomoniasis the potential ability of T. vaginalis to cling to the epithelial surfaces of the vagina or male urethra offers the organism a means of resisting removal from the sites of infection in any outflowing discharge. Thirdly the cytopathogenic mechanisms of the parasite will be of greater effect if they are applied



at close range by an organism to a cell to which it is adhering than if that organism was only swimming freely at some distance from the cell.

#### 4.4 The amoeboid movements of *T. vaginalis*

It may come as a surprise to some people that *T. vaginalis* can move on a solid substratum like an amoeba. Textbooks of parasitology commonly describe the organism as an ovoid protozoon equipped with four anterior flagella and an undulating membrane which enable it to swim through liquids, and so it is perhaps understandable that little attention has been paid in the past to the possibility that *T. vaginalis*, a flagellate, may possess an alternative mode of locomotion.

This is the first detailed study of the amoeboid movements of *T. vaginalis*. I have made use of time-lapse still photomicrography to show the motility of the organism on glass; undoubtedly future studies using time-lapse cinemicrography will be of greater value in elucidating the finer points of the behaviour of amoeboid *T. vaginalis*. In fact both Hogue (1947) and Christian et al. (1963) made movie films of cell cultures infected with *T. vaginalis* but so far as I am aware they did not use their films to analyse the motility of the parasite.

##### 4.4.1 General characteristics

Some of the features of the amoeboid movements of *T. vaginalis* are similar to those of many true amoebae, i.e. members of the class Rhizopodea (Honigberg et al. 1964). Typically, the ability rapidly to protrude and retract pseudopodia and filopodia, the optically uniform density and lack of organelles of the peripheral parts of the pseudopodia, and the ability to move either as a monopodial or multipodial form, are features which amoeboid *T. vaginalis* share with, for example, *Acanthamoeba* spp., *Naegleria* spp., (Griffin, 1978) *Entamoeba* spp. (Albach and Booden, 1978) and *Amoeba proteus* (Jeon, 1973).

An amoeboid *T. vaginalis* moves at a speed of about 20  $\mu\text{m}$  per

min., thus its amoeboid motility is comparable to that of a similar-sized amoeba such as Naegleria fowleri which moves at a speed of 28 to 37  $\mu\text{m}$  per min (Griffin, 1978). The changes in the direction of movement of T. vaginalis appeared to be fairly randomly spaced over a given period of time however the monopodial forms often persisted longer in moving in one direction than the multipodial forms. This raises the intriguing question as to whether the direction of movement of T. vaginalis may be influenced by a chemical gradient, i.e. chemotactically. It is noteworthy that Hogue (1943) observed that T. vaginalis was "attracted by some fluid" exuding from cultured explants of human tissues infected with the parasite. Many other protozoa exhibit chemotaxis (Westphal, 1976) and such a behavioural characteristic, if shown in T. vaginalis, could have profound implications in the biology of the parasite.

#### 4.4.2 Mechanism of amoeboid movements of T. vaginalis

As mentioned earlier, since T. vaginalis can crawl like an amoeba on a substratum it must possess an internal contractile system capable of generating the forces necessary to pull its body towards its anterior adhesions with the substratum and also capable of projecting pseudopodia in front of the body so that they may make new adhesions to the substratum.

The pseudopodia of T. vaginalis contain a meshwork of microfilaments, about 5 nm in diameter and of variable lengths, some of which are arranged into bundles up to 3  $\mu\text{m}$  long. These microfilaments are morphologically similar to the filamentous polymerised form of the protein actin, known as F-actin (Pollard, 1973). The sliding interaction of actin and myosin forms the molecular basis for contraction and force generation in metazoan skeletal muscle (Huxley, 1973). It is now widely recognised that actin- and myosin-like proteins are also present in many, if not all, metazoan non-muscle cells (Huxley, 1973), in rhizopod amoebae, such as A. proteus and Acanthamoeba castellanii



(Pollard, 1976), in slime molds (Wohlfarth-Bottermann and Isenberg, 1976), and in characean algae (Williamson, 1976). The discovery of actins and myosins in animals, protists and plants suggests that actomyosin systems developed very early in evolutionary terms and that muscle contraction is a highly specialised example of a more generalised mechanism of producing movement in cells (Pollard, 1973; Huxley, 1973).

In view of the morphological similarities of the microfilaments of T. vaginalis to those of the rhizopod amoebae (which are known to be composed of F-actin [Pollard 1976]) and the similarities of the amoeboid movements of T. vaginalis with those of amoebae (which incidentally illustrates the basic evolutionary affinities of general flagellates and amoebae [Honigberg et al., 1964]) it seems not unreasonable to presume that the amoeboid movements of T. vaginalis may have the same molecular basis as those of the rhizopod amoebae. Indeed recent evidence, although much of it only circumstantial, suggests that the cytoplasmic movements of many protozoa, other than amoebae, may also involve actomyosin systems. Tucker (1978) discovered a ring of microfilaments in the cytopharyngeal basket of the ciliate Nassula which appeared to be involved in the closure of the basket after the ingestion of food. The gregarine Zeylanocystis burti has bundles of microfilaments deep within its cytoplasm which are most clearly evident when the parasite is flexed, implying an involvement of the microfilaments in torsion of the body (Sathananthan, 1977). Bundles or meshworks of 3 to 5 nm diameter microfilaments have been observed in several kinetoplastid flagellates (see review of Vickerman and Preston, 1976). In all these protozoans, and in T. vaginalis, the evidence that the microfilaments are actin rests on morphological similarity only and needs to be confirmed by biochemical tests or by showing that the filaments bind heavy meromyosin (Pollard, 1973) or antibodies to actin. This latter test was used by Erlandsen et al. (1978) on Giardia muris;

they showed, using gel electrophoresis and immunofluorescent antibody staining, that the fibrous masses associated with the adhesive disc, axonemes and axostyle of the parasite contained actin- and myosin-like proteins, and they suggested that an actomyosin system was involved in shape changes of the adhesive disc.

It is possible therefore that the microfilament meshworks of the pseudopodia of T. vaginalis may by contracting and squeezing the cytoplasm of the parasite cause the extension of the pseudopodia over the substratum during locomotion, or around an ingestible object during phagocytosis. In a similar way the bundles of microfilaments which often lay perpendicularly to the points of contact of the pseudopodia with the epithelial cells, the glass substratum or other trichomonads, could by contracting draw the body of the organism into closer contact with the surface to which it is adhering. Whatever the ways in which the microfilaments of T. vaginalis are involved in movement, it is clear that the organism must also possess a mechanism controlling the appearance of the filaments since I found none in non-phagocytosing T. vaginalis prepared directly for EM from axenic cultures whereas they were always present in the pseudopodia of the amoeboid organisms which were adhering to a surface. This suggests that it is only when T. vaginalis comes into contact with a surface that the microfilaments are synthesised and operate. It may be that the cytoplasm of a non-phagocytosing trichomonad which is swimming freely in suspension contains a pool of G-actin, the soluble monomeric form of actin, and that when part of its surface makes contact with an object, whether it be a food particle or a substratum, the contact triggers the polymerisation of G-actin into F-actin, in the form of a microfilament meshwork, which by interacting with other contractile proteins such as myosin results in the extension of pseudopodia over the object. Only further experimentation will elucidate the molecular basis of amoeboid motility in T. vaginalis.



#### 4.4.3 Importance of amoeboid motility in the biology of *T. vaginalis*

The ability of *T. vaginalis* to move either as a flagellate, using its flagella and undulating membrane, or as an amoeba, employing pseudopodia, or indeed using both mechanisms, must be of advantage to a parasite of the human urogenital tract which may at times be surrounded by fluids of varying viscosity. For example, *T. vaginalis* may use its flagella to move about the urethra if there is a thin film of urine present; however if the parasite is surrounded by a highly viscous and purulent discharge, such as is common in florid trichomonal vaginitis, and in which its flagella may not operate properly, then amoeboid locomotion using either the vaginal epithelium or the cells within the discharge as a substratum would be a more efficient mode of movement.

The fact that *T. vaginalis* can adopt an amoeboid morphology quite different from the spherical or ovoid form typical of the organism when suspended in a liquid suggests that to the untrained eye the parasite could easily be mistaken for an epithelial cell or leucocyte in a wet film preparation of urogenital secretion, a technique widely used in the diagnosis of trichomoniasis in human, thus leading to errors in diagnosis. Textbooks on venereal diseases such as those of Schofield (1972) and King and Nicol (1975) make no mention of amoeboid forms of *T. vaginalis* so clearly this is a situation which could be remedied.

#### 4.5 Mechanisms of pathogenicity of *T. vaginalis*

Earlier studies showed that parasite-free filtrates of *T. vaginalis* cultures contained substances which were injurious to cultured cells (Hogue, 1943; Farris and Honigberg, 1970). Culture filtrates have also been shown to cause the haemolysis of human and rabbit erythrocytes by Grys and Hernik (1973) however these authors did not make it clear if haemolysis was due to the presence of lytic agents in the filtrates or simply due to a possible hypotonicity of the filtrates. Nevertheless these observations suggest that the parasite secretes cytotoxic factors

into its environment which cause the pathological changes observed in trichomonal infections both in vivo and in vitro. However other workers, unable to detect any cytopathogenic effects of culture filtrates on cells, argued that T. vaginalis mechanically injures cells as a result of the ability of the organism to adhere to, and perform amoeboid movements on, cell surfaces (Kotcher and Hoogasian, 1957; Christian et al., 1963; Kulda, 1967).

The data presented in this study when added to the large volume of previous work on T. vaginalis enables us to postulate some mechanical and chemical mechanisms which may be involved in the cytopathogenicity of T. vaginalis in cell cultures and in humans.

#### 4.5.1 Mechanical mechanisms

The results of this study suggest several ways in which T. vaginalis could mechanically injure tissues and cells. In the RK 13 cell cultures amoeboid T. vaginalis were able to migrate under the monolayer of cells indicating that the protrusive activities of the pseudopodia of the parasites provided the necessary force to break the adhesions of the cells to the substratum. Although one might expect the cells of a tissue to be strongly cohesive in order to maintain its integrity, it is well known that the adhesions between cells do not always prevent the passage of another cell between them as, for example, in the diapedesis of leucocytes between the endothelial cells lining capillary walls. Of more direct relevance to this study is the report of Middleton (1973) who found that when chick fibroblasts were placed on top of a confluent sheet of cultured chick retinal epithelial cells, which as far as their intercellular and cell-substratum adhesions are concerned were similar to a confluent monolayer of RK 13 cells, the fibroblasts penetrated the sheet and came to lie between the lower surfaces of the epithelial cells and the substratum, i.e. in a similar position as the amoeboid trichomonads in RK 13 cell cultures. Thus if sufficient numbers of motile parasites migrated into an area of the human vaginal epithelium they could



disrupt the mutual adhesions of the superficial epithelial cells causing erosion of the epithelium. This mechanism may account for the occurrence of shallow depressions in the superficial layers of the vaginal epithelium found in some cases of human trichomoniasis (Nielsen and Nielsen, 1975).

A second way in which T. vaginalis may mechanically damage cells is by nipping off pieces of cell cytoplasm thus rupturing the plasma membrane of the cell and causing the leakage of soluble cytoplasmic material. In the RK 13 cultures I found several instances when the pseudopodia of T. vaginalis appeared to be nipping off peripheral projections and microvilli of the cells. A similar phenomenon was observed by Farris and Honigberg (1970) in the regions of contact between T. vaginalis and chick macrophages, and by Tamayo et al. (1972) at contacts between the parasite and human vaginal epithelial cells. Mechanical rupture of the plasma membrane of a cell to which T. vaginalis is adhering may also arise as a result of the tensions applied to the cell surface as the organism moves over the cell. Using SEM I found cases when amoeboid T. vaginalis were stretched out on the surface of RK 13 cells, and both I and Nielsen and Nielsen (1975) have shown that bundles of putatively-contractile microfilaments pass into the cytoplasm of the organism from its adhesions with another cell or a solid surface. On a non-deformable substratum such as glass the forces generated by the microfilament bundles would tend to draw the body of T. vaginalis towards the adhesions; however if the adhesions are to a deformable substratum such as the plasma membrane of a cell the opposite may occur and the cell surface may be drawn towards the parasite and physically damaged.

Another way in which T. vaginalis may mechanically cause cell injury is suggested from the work of Munro and Daniel (1965) on the lethal effects of nuclear membrane rupture in cells. They found that

the insertion of a 3  $\mu$ m diameter tungsten needle into the nucleus of cultured hamster fibroblasts was always lethal, resulting in an increase in phase density of the nuclear membranes and chromatin, rounding up of mitochondria, ballooning of the cytoplasm and death of the cells within 15 min. Insertion of the needle into the cell cytoplasm had little or no injurious effects unless the plasma membrane was torn by lateral movements of the needle in which case the cell died, possibly as a result of a large change in cytoplasmic tonicity. It is conceivable therefore that the axostyle of T. vaginalis, which is, judging by its straightness, a fairly rigid structure, may penetrate cell plasma membranes and nuclear membranes causing injury. However, although T. vaginalis adhered to the epithelial cells by its axostyle there was no evidence in my electron micrographs of the axostyle penetrating cells. If indeed the axostyle can injure cells, such an occurrence may be more common when the parasite is lying deep in the tissues of a host where pressure from adjacent host cells or from the motility of the parasite may aid penetration. On the other hand I did observe several instances when pseudopodial projections from amoeboid T. vaginalis made deep indentations into the surface of epithelial cells in regions close to the nucleus; although there is no evidence to suggest that the pseudopodia of T. vaginalis are rigid, on the contrary I found that they appeared to be fairly flexible, they could exert injurious pressure on the sensitive nuclear membrane.

Attractive as the above mechanisms may be it will be extremely difficult to prove conclusively that they operate in the absence of additional chemical factors secreted by T. vaginalis.

#### 4.5.2 Chemical mechanisms

Two possible sources of cytotoxic factors secreted by T. vaginalis are: 1) the main end products of carbohydrate metabolism, lactic acid, acetic acid, CO<sub>2</sub> and H<sub>2</sub> (Honigberg, 1978b), and 2) catabolic enzymes,



such as the hydrolases present in the phagosomes and lysosome-like vesicles of T. vaginalis (Lindmark et al., 1975).

#### 4.5.2.1 Metabolic waste products

Taylor (1962) has shown that cultured cells are sensitive to an increase in acidity of the culture medium; changing the pH of the medium of cultured avian and human epithelial cells from 7.3 to 5.6 caused a cessation of cell movement, an increase in cytoplasmic and nuclear granularity and eventual cell lysis after 5 h. Although T. vaginalis secretes lactic and acetic acid into its environment it is unlikely that acidity played any role in the initial stages of the infection of RK 13 cultures with T. vaginalis since I found that the medium of the cultures had a pH of about 6.5 six hours after inoculation of the parasites by which time there were many lesions. Farris and Honigberg (1970) also considered the possible injurious effects of low pH in chick liver cell cultures infected with T. vaginalis but discounted them when they found that 24 h post-infection, when all the cells were destroyed, the culture medium was pH 6.0 compared to 6.7 at the start. However in the later stages of my T. vaginalis-infected RK 13 cultures, i.e. phases 4 and 5, the acidity increased to pH 5.0 or less and so the metabolic products of the parasites, and also a probable concomitant decrease in nutrients in the culture medium, probably contributed to the death and lysis of the remaining cells. However, in natural infections in females it is not clear how metabolic waste products of T. vaginalis could be involved in pathogenicity since acidic conditions (about pH 4) prevail in the normal human vagina.

#### 4.5.2.2 Enzymes

Earlier biochemical and cytochemical studies on T. vaginalis (see below) and other members of the Trichomonadida such as Monocercomonas sp. (Lindmark and Muller, 1974) and Tri. foetus (Muller, 1973) have shown that these trichomonads possess many acid and neutral hydrolytic

enzymes which are involved in the intracellular digestion of ingested particulate and soluble material (see also reviews of Eekhout, 1973; Honigberg, 1978a, 1978b).

Acid phosphatase is present in the Golgi body, in the 50 to 100 nm diameter membrane-bound vesicles derived from the Golgi, in phagosomes and in pinosomes of T. vaginalis (Ohashi, 1972; Tamayo et al., 1972; Nielsen, 1974; Lindmark, et al., 1975; this study). These organelles and particles also contain neutral ATP-ase (Nielsen, 1974) and  $\beta$ -N-acetylglucosaminidase (Lindmark et al., 1975). Sharma and Bourne (1964) using a light microscopic cytochemical method detected  $\beta$ -glucuronidase activity in the cytoplasmic granules of T. vaginalis however both Fishman et al. (1950) and I, in this study, have not been able to confirm the presence of this enzyme from biochemical assays on homogenates of the organism. Although further work is necessary to characterise the subcellular distribution of hydrolytic enzymes in T. vaginalis and to discover if stocks of the parasite differ in their enzyme content, all the present evidence suggests that T. vaginalis possesses a lysosomal system, similar to that of metazoan cells (De Duve and Wattiaux, 1966), in which hydrolase-containing vesicles or primary lysosomes are formed at the Golgi body and then fuse with and release their contents into phagosomes so forming secondary lysosomes in which nutrients are digested.

It is possible therefore that exocytosis of the digested remains of the contents of the phagosomes, or perhaps exocytosis of the enzymes in primary lysosomes, with the consequent release of digestive enzymes into the environment of T. vaginalis may be related to the cytopathogenicity of the parasite. In this study I found that the cytoplasm of T. vaginalis at the areas of adhesion to the RK 13 cells contained enzymes, which may have been en route to the plasma membrane to undergo exocytosis so releasing hydrolytic enzymes which could digest the surfaces of the RK 13 cells.



Other enzymes which may be considered as possible factors in the pathogenicity of T. vaginalis are hyaluronidase, neuraminidase and phospholipase.

Boni and Orsi (1958) and Filadoro and Orsi (1960) using a viscometric assay showed that both the living organisms and homogenates of 8 strains of T. vaginalis were able to digest bovine sinovial fluid and human hyaluronic acid indicating the presence of the enzyme hyaluronidase in the parasites. Hyaluronic acid occurs widely as an intercellular mucopolysaccharide of metazoan tissues; secretion of hyaluronidase by T. vaginalis may facilitate the invasion of host tissues by amoeboid forms of the parasite by breaking down intercellular cements.

Neuraminidases are secreted by several different parasites of mucosal surfaces, such as viruses and bacteria (Haskell, Peterson, Watson, Plessas and Culbertson, 1970) and Tri. fetus (Romanovska and Watkins, 1963). Mucosal secretions are rich in sialic acids and these acidic sugars may be involved in maintaining the viscosity of secretions (Haskell et al., 1970); neuraminidase selectively removes sialic acids from glycoproteins thus parasites which secrete this enzyme possess a mechanism for penetrating the protective mucosal secretions lining the respiratory and urogenital tracts. Muller and Saathof (1972) consider that the secretion of neuraminidase by Tri. fetus enables the parasite to degrade the mucus of the bovine cervical surface so permitting invasion of the uterus (the typical habitat of this parasite in bovine trichomoniasis) with the result that Tri. fetus may cause abortion and infertility in cattle. I was unable to detect neuraminidase in homogenates of T. vaginalis and if the absence of this enzyme in the organism is confirmed by future studies on many other stocks of T. vaginalis it may partly explain why the uterus is not infected in cases of human trichomoniasis.

Recent studies have indicated that the cytopathogenicity of

Entamoeba histolytica and E. invadens (McCaul, Poston and Bird, 1977) and Naegleria fowleri and Acanthamoeba culbertsoni (Cursons and Brown, 1978) may be related to the injurious effects of phospholipases of these amoebae on the plasma membranes of their host cells. As far as I am aware no-one has looked for phospholipases in T. vaginalis so this could be an avenue worthy of investigation in view of the similar cytopathogenic effects which occur in RK 13 epithelial cell cultures when infected with T. vaginalis (this study) or E. histolytica (Knight et al., 1975).

In summary, it is clear that further work is needed on the characterisation of the enzymes of T. vaginalis and their subcellular distribution before we can draw any firm conclusions concerning their relationship to the cytopathogenicity of the parasite. In addition we need to know which, if any, of the metabolic enzymes of the organism are involved in extracellular digestion; an analysis of the parasite-derived enzyme content of filtrates of T. vaginalis cultures may provide some of the answers to the problems of chemical mechanisms of pathogenicity of this fascinating parasite.



## 5. CONCLUSIONS

1. T. vaginalis can adhere to a variety of living and non-living surfaces. The adhesions between the organism and a surface are generally of the intermediate-junction type and characterised by a gap of 10 to 30 nm.
2. In natural human infections the ability of T. vaginalis to adhere to the epithelial surfaces of the urogenital tract may be a mechanism which enables the parasite to resist removal from its host by outflowing discharges or urine.
3. T. vaginalis can move either as a flagellate or like an amoeba. Amoeboid motility is possible because T. vaginalis possesses an internal force-generating system, probably based on actin-like microfilaments, that is capable of being assembled and organised on contact of the surface of the organism with a substratum or an ingestible object, and this system provides the mechanism for the protrusion of pseudopodia over the substratum during amoeboid locomotion or around the object during phagocytosis.
4. In natural infections the amoeboid motility of T. vaginalis would enable the parasite to move about within its host in situations where its flagella and undulating membrane cannot operate efficiently as locomotory organelles, such as in viscous vaginal discharge and in spaces between the cells of host tissue.
5. T. vaginalis infections of monolayers of cultured mammalian epithelial cells provide a valuable experimental method for studying the inherent mechanisms of pathogenicity of the parasite at the light and electron microscope levels. The lesion-forming ability of the parasite in cell monolayers may with suitable developments prove to be the basis for an efficacious method of assaying the

pathogenicity of stocks of T. vaginalis.

6. The cytopathogenicity of T. vaginalis probably involves a combination of mechanical and chemical mechanisms. Amoeboid forms of the parasite may mechanically damage tissues and cells by breaking the adhesive junctions between cells, and between cells and their substrata, and by rupture of the plasma membrane of cells with their pseudopodia or axostyles. T. vaginalis may chemically injure cells by secreting toxic waste products or hydrolytic enzymes such as acid phosphatase, ATP-ase,  $\beta$ -N-acetyl-glucosaminidase and hyaluronidase.
7. Chemicals or drugs which inhibit the adhesiveness and amoeboid motility of T. vaginalis may be valuable tools for distinguishing the contribution of mechanical mechanisms, which depend on adhesion and motility, from that of chemical mechanisms in the pathogenicity of T. vaginalis.
8. Differences in the inherent pathogenicity of strains and stocks of T. vaginalis may be related to differences in their adhesiveness and abilities to perform amoeboid movements.



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TABLE 1

Sexually transmitted diseases in England 1973-1977; new cases per 100,000 population at all ages seen in hospital clinics (data taken from "On the state of the Public Health", Department of Health; London: H.M.S.O. (1978))

Years ending 30 June															
	1973			1974			1975			1976			1977		
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
Trichomoniasis	7.08	72.39	40.02	6.88	74.02	41.35	6.42	74.96	41.58	6.81	76.23	42.42	7.54	76.86	43.09
Candidiasis	20.42	108.17	65.49	22.84	115.53	70.42	23.71	116.83	71.48	27.68	125.84	78.02	29.89	125.99	79.18
Gonorrhoea	157.30	80.94	118.08	169.60	88.92	128.19	163.67	87.62	124.66	163.91	89.60	125.79	164.15	92.00	127.15
Non-specific genital infection	286.34	60.01	170.10	307.95	63.58	132.50	306.90	65.87	183.25	310.35	73.60	188.91	327.40	84.72	202.95



TABLE 2

Some details of previous studies on T. vaginalis-infected cell cultures

<u>Specifications of Cell Cultures</u>		<u>T. vaginalis</u>	<u>Pathological changes</u>	<u>Time taken for cultures to be totally destroyed, and density of parasites at this time or at end of experiment.</u>
1. Tissue type	2. Cell type	1. Strain designation	and times post-inoculation when changes occur	
3. Culture conditions	NG = Not given	2. Authors' evaluation of pathogenicity level		
		3. No. of parasites per culture		
<u>Christian et al. (1963)</u>		1. NG	No times and very few details given.	60 h
1. Human cervical carcinoma (HeLa)		2. NG	Cell-free areas (lesions) appear in monolayer; lesions lined by degenerating cells. Parasites detach cells from coverslips. Lesions enlarge until all cells destroyed	1 x 10 <sup>6</sup> parasites per culture
2. Epithelial cells		3. 5 x 10 <sup>3</sup>		
3. Confluent monolayer of cells on 25 mm diam. circular coverslips.		1. NG	Very few cytopathological changes	3.5 x 10 <sup>3</sup> parasites per culture at 60 h.
Eagle's cell culture medium.		2. NG		
Volume of medium NG.		3. 5 x 10 <sup>2</sup>		
<u>Kulda (1967)</u>		1. 108	12 h post inoculation: Cell injuries apparent. Decrease in division rate of cells; cells vacuolated with abnormal nuclei; cells lose their normal shape and retract. Lesions in monolayer. 20 h: many cells damaged, large lesions in cell sheet. 24 h: large parts of monolayer peeled off glass	48 h 5.5 x 10 <sup>5</sup> parasites per ml
1. Monkey kidney		2. High		
2. Fibroblasts		3. 4 x 10 <sup>5</sup>		
3. Confluent monolayer of cells on 10 x 20 mm coverslips. 2 ml of cell culture medium 199.		1. 101	Changes much the same as above but the destruction of the monolayer runs a slower course.	60 h 6.5 x 10 <sup>5</sup> parasites per ml
		2. Low		
		3. 4 x 10 <sup>5</sup>		

continued on next page

TABLE 2 cont.

<u>Specifications of Cell Cultures</u>	<u>T. vaginalis</u>	<u>Pathological changes</u>	<u>Time taken for cultures to be totally destroyed.</u>
<u>Farris and Honigberg (1970)</u>			
1. Chick embryo liver	1. JH30A	2 h: Small lesions 200 µm in diam. in monolayer	24 h
2. Epithelial cells, fibroblasts and macrophages	2. High	8 h: Distinct morphological abnormalities in all cell types; cytoplasm of cells retracted, cells vacuolated, nuclei often pyknotic, 50 per cent inhibition of division	3 x 10 <sup>5</sup> parasites per culture
3. Confluent monolayer of cells on 9 x 22 mm coverslips. 1 ml of a 2:1 mixture of cell culture medium and <u>Trichomonas</u> medium	3. 1 x 10 <sup>5</sup>	20 h: cultures riddled with lesions, cells peeling off glass	
	1. JH32A	Very few changes evident before 8 h.	8 x 10 <sup>5</sup> parasites per culture
	2. Low	8 h to 24 h: Degenerative changes similar to those caused by JH30A strain but much less extensive. Cultures not destroyed at 24 h.	
	3. 1 x 10 <sup>5</sup>		



TABLE 3

Details of *T. vaginalis* stocks

LUMP No.	Patient No.	Isolation date.	TV	Wet film SEC	Wet film <sup>1</sup> Pus	Duration of symptoms <sup>2</sup>	Concurrent STD. <sup>3</sup> none = -	Use of oral contraceptives Yes = +; No = -	Days in culture before cryopreservation	Cryopreservation date
840	963	14.2.73	3	2	3		C, P		2	16.2.73
843	1012	16.2.73	3	2	3	2 m	-	+	3	19.2.73
846	1040	19.2.73	3	2	3	2 m	-	-	2	21.2.73
847	1083	20.2.73	3	2	3	1 w	C, Sc	+	3	23.2.73
849	1109	21.2.73	4	2	3	1 w	-	-	2	23.2.73
851	1095	21.2.73	2	2	2	2 d	G	-	6	27.2.73
852	1137	22.2.73	0	2	3	3 w	-	+	7	1.3.73
853	1141	22.2.73	0	2	2		-		7	1.3.73
863	1328	5.3.73	2	1	1	AS	C	-	7	12.3.73
864	1329	5.3.73	1	2	3	3 w	-	+	4	9.3.73
865	1354	6.3.73	2	2	3	4 d	-	+	3	9.3.73
866	1460	12.3.73	3	1	3	3 w	W	-	2	14.3.73
869	1485	13.3.73	2	1	3	2 w	M	+	3	16.3.73
870	1519	14.3.73	3	2	3	3 w	G	-	6	20.3.73
871	1803	28.3.73	2	2	3	3 w	G, C	-	2	30.3.73
872	1824	29.3.73	2	2	3	6 w	-	+	1	30.3.73
873										
878	1838	29.3.73	3	2	3	1 w	-	+	4	2.4.73
881	1944	4.4.73	2	1	3		-		6	10.4.73
882	2154	13.4.73	2	3	2	2 d	-	-	3	16.4.73

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continued on next page

TABLE 3 continued

LUMP No.	Patient No.	Isolation date.	TV	Wet film <sup>1</sup> SEC Pus	Duration of symptoms <sup>2</sup>	Concurrent STD. <sup>3</sup> none = -	Use of oral contraceptives Yes = +; No = -	Days in culture before cryopre- servation	Cryopreserva- tion date
884	2112	12.4.73	0	1 2				5	17.4.73
885	2184	16.4.73	2	1 1	7 m	-	-	2	18.4.73
886	2205	17.4.73	3	3 2	AS	C, NSU	+	2	19.4.73
887	2178	16.4.73	0	2 2	2 m	-	+	9	25.4.73
888	2207	17.4.73	2	3 2	AS	-	-	8	25.4.73
889	2236	18.4.73	4	2 3	AS	G	+	6	24.4.73
890	2238	18.4.73	2	1 3	1 w	G Sc	-	7	25.4.73
893	2351	26.4.73	4	1 3	AS	G	+	4	30.4.73
894	2372	27.4.73	0	1 1	1 w	H	-	5	2.5.73
895	2494	3.5.73	0	1 2		G		4	7.5.73
896	2512	4.5.73	0	1 3		-		3	7.5.73
900	2521	4.5.73	0	3 1	AS	-	+	5	9.5.73
901	2551	7.5.73	4	2 2	12 m	C	-	2	9.5.73
904	2638	10.5.73	0	2 3		-		4	14.5.73
905	2633	10.5.73	0	3 3	AS	G	-	6	16.5.73

## Notes.

1. Arbitrary scale of the numbers of trichomonads (TV), squamous epithelial cells (SEC) and leucocytes (Pus) seen in the wet film examination of each secretion. For explanation see 2.2.1.

2. d = days w = weeks m = months AS = asymptomatic

3. Concurrent sexually transmitted disease (STD)

G = gonorrhoea, C = candidiasis, H = herpes genitalis, W = genital warts, Sc = scabies, M = molluscum contagiosum.

P = pubic lice

Blank spaces indicate data not available



TABLE 4

Acid phosphatase assay of T. vaginalis

<u>Fraction of homogenate of T. vaginalis assayed</u>	<u>Specific Activity of acid phosphatase</u> nanoMoles paranitrophenol released per $\mu\text{g}$ <u>T. vaginalis</u> protein per min. at 37°C	
	<u>T. vaginalis</u> LUMP 889 $1.25 \times 10^8$ cells <sup>1</sup>	<u>T. vaginalis</u> LUMP 993 $2 \times 10^7$ cells <sup>1</sup>
1) Soluble fraction of the cytoplasm = cytosol	$32.7 \times 10^{-3}$	$3.64 \times 10^{-3}$
2) 105,000 g sedimentable particle fraction		
a) Free activity	$140.1 \times 10^{-3}$	$80.2 \times 10^{-3}$
b) Activity releasable by Triton X-100 solubilisation	$40.9 \times 10^{-3}$	$123.6 \times 10^{-3}$
c) Total activity	$181.0 \times 10^{-3}$	$203.8 \times 10^{-3}$
Ratio of enzyme activities, particle fraction:cytosol	5.5:1	55:1

1. Number of organisms from which each fraction of the homogenate was obtained.

TABLE 5

Neuraminidase assay of T. vaginalis

<u>Material assayed</u> <sup>1</sup>	<u>Optical density</u> <sup>2</sup>	<u>%</u> <sup>3</sup>
<u>Parasites</u>		
1) <u>T. vaginalis</u> LUMP 840:		
a) $5 \times 10^5$ living organisms	0.000	0.0
b) Cytosol of $5 \times 10^5$ lysed organisms	0.000	0.0
2) <u>T. vaginalis</u> LUMP 896:		
a) Cytosol of $5 \times 10^6$ lysed organisms	0.001	0.26
b) Cytosol + solubilised particles of $5 \times 10^6$ lysed organisms	0.013	3.44
<u>Controls</u>		
3) Neuraminidase (BDH Ltd) 250 International units in $H_2O$	0.378	100.0
4) Neuraminidase (Wellcome Ltd) 1:10 dilution in $H_2O$	0.136	36.0
5) 0.1 per cent aqueous soln. sialomucoids + 0.4 N $H_2SO_4$ incubated at 80°C for 30 min before assay	0.236	62.4
6) $H_2O$	0.010	2.6

1. Volume of each material = 0.5 ml. Each material was added to an incubation vessel containing 0.5 ml 0.2 M acetate buffer, pH 6.1, and 1 ml 0.1 per cent aqueous soln. of sialomucoids; the mixtures were incubated at 37°C for 30 min., with the exception of material (5) when the sialomucoids soln. was replaced with 1 ml  $H_2O$ . For the assay procedures and the determination of optical density see 2.7.5.
2. Optical density is directly proportional to the concentration of sialic acid released from the sialomucoids by each of the test materials. Value given is the mean of 2 experiments.
3. Neuraminidase activity of material as a percentage of the activity of 250 IU of neuraminidase in (3).



.....  
FIGURES

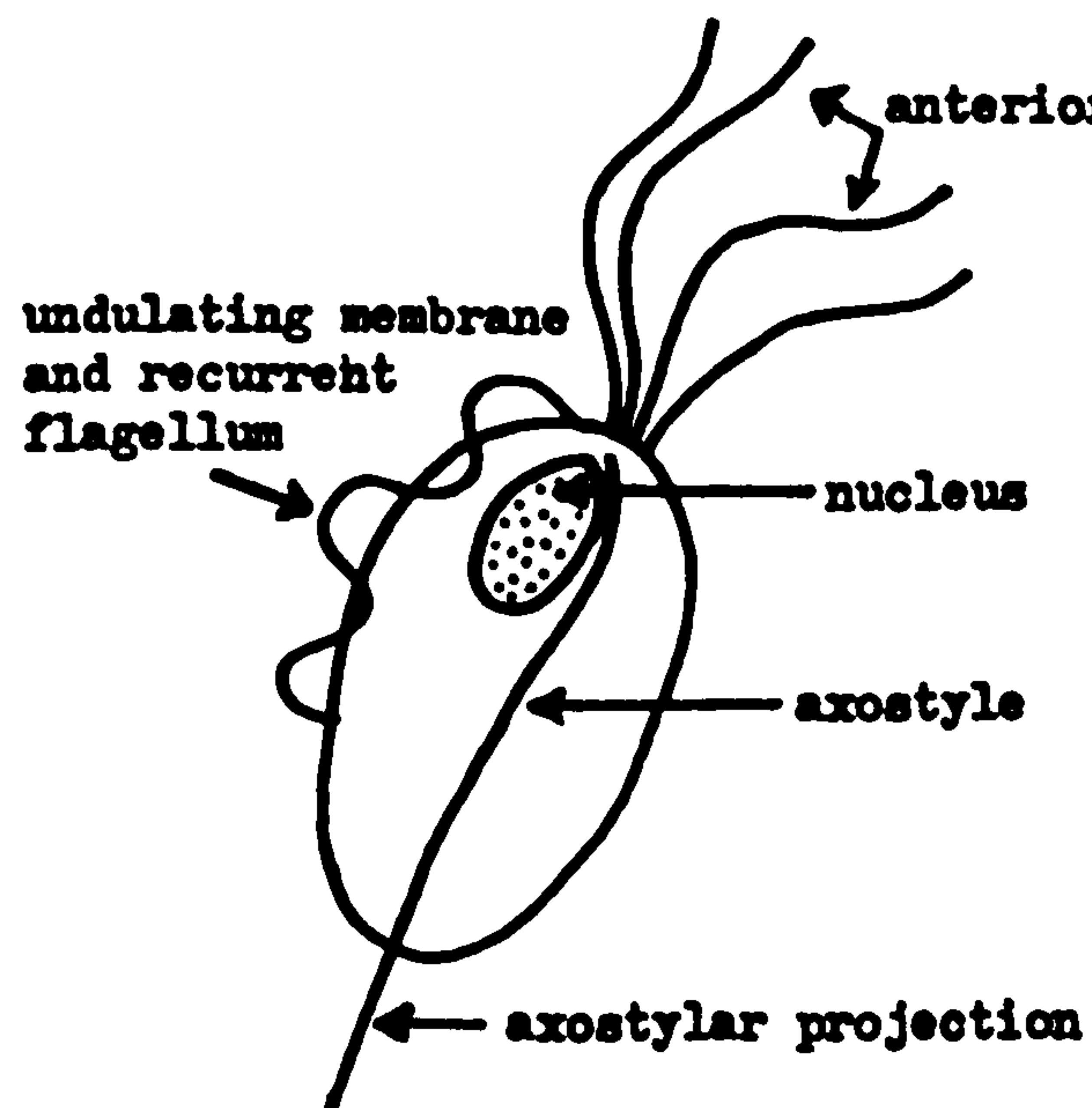
Main abbreviations used on figures, for others see figure legends.

af	anterior flagellum or flagella
ax	axostyle
c	costa
ec	epithelial cell
ecm	epithelial cell monolayer
er	endoplasmic reticulum
f	flagellum or flagella
fi	filopodium
fp	flagella pocket
g	Golgi body
gl	glycogen granule
h	hydrogenosome
HS	horizontal section, cut parallel with the substratum
ij	intermediate-type junction
IR	interference reflection
k	kinetosome
L	lesion
la	lamellar cytoplasmic extension
m	mitochondrion
med	medium-facing side of monolayer
mf	microfilaments
ml	marginal lamella
mv	microvillus
n	nucleus
nl	nucleolus
p	pseudopodium
PC	phase contrast
pe	pelta
pf	parabasal filament
ph	phagosome
pm	plasma membrane
rf	recurrent flagellum
rt	retraction fibre
s	substratum
t	microtubule
tj	tight junction
tv	<u>T. vaginalis</u>
um	undulating membrane
v	cytoplasmic vesicle
va	cytoplasmic vacuole
VS	vertical section, cut perpendicular to the substratum

Fig. 1a. Some members of the order Trichomonadida. Original drawings based on the descriptions given by Honigberg (1978a, 1978b), showing the main organelles. T. vaginalis and T. gallinae are morphologically similar. Each member is drawn to the same relative scale as they appear in stained preparations from axenic cultures; however it must be noted that there is often a wide variation in the sizes of the individuals of any population of trichomonads.

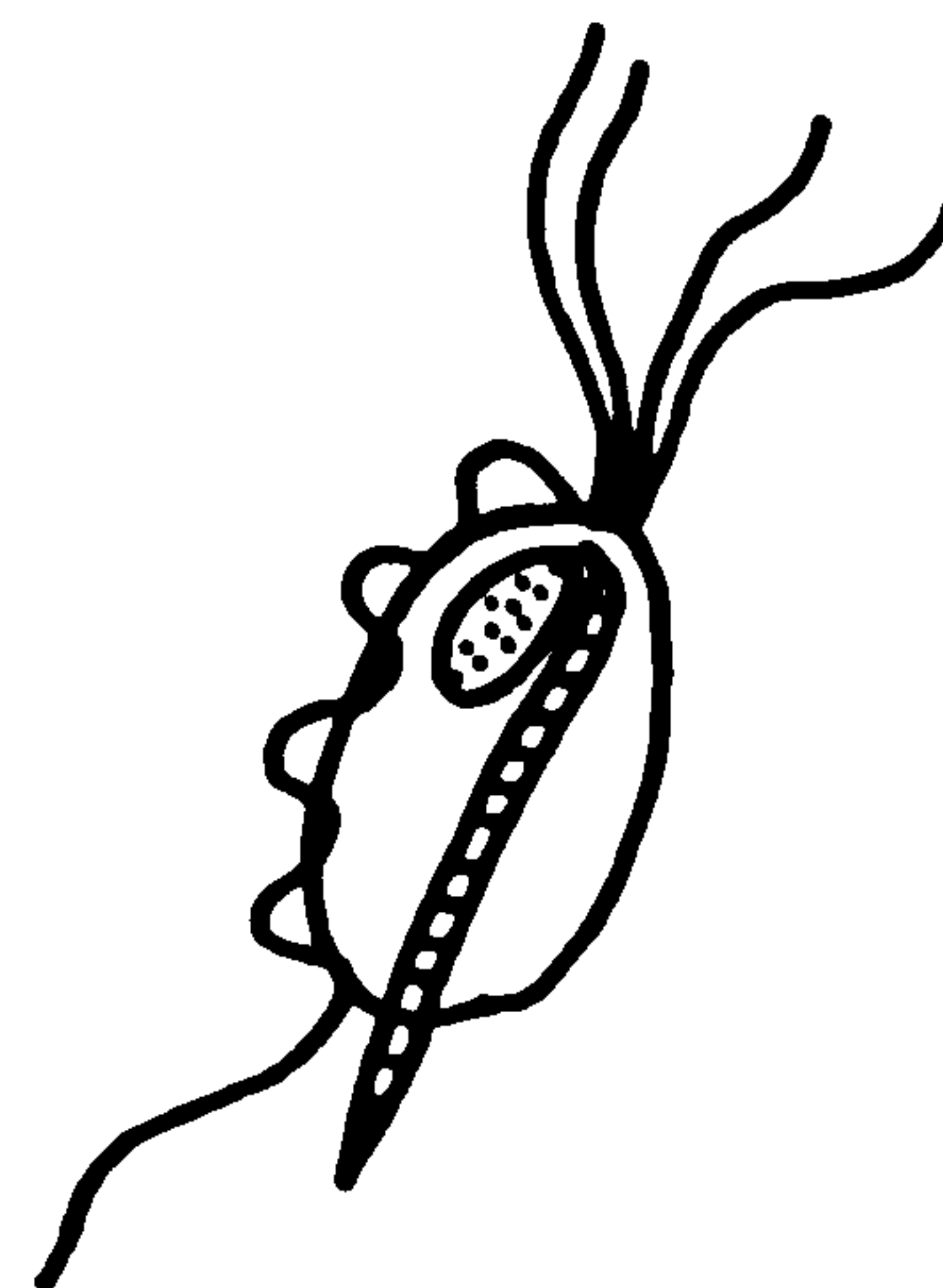
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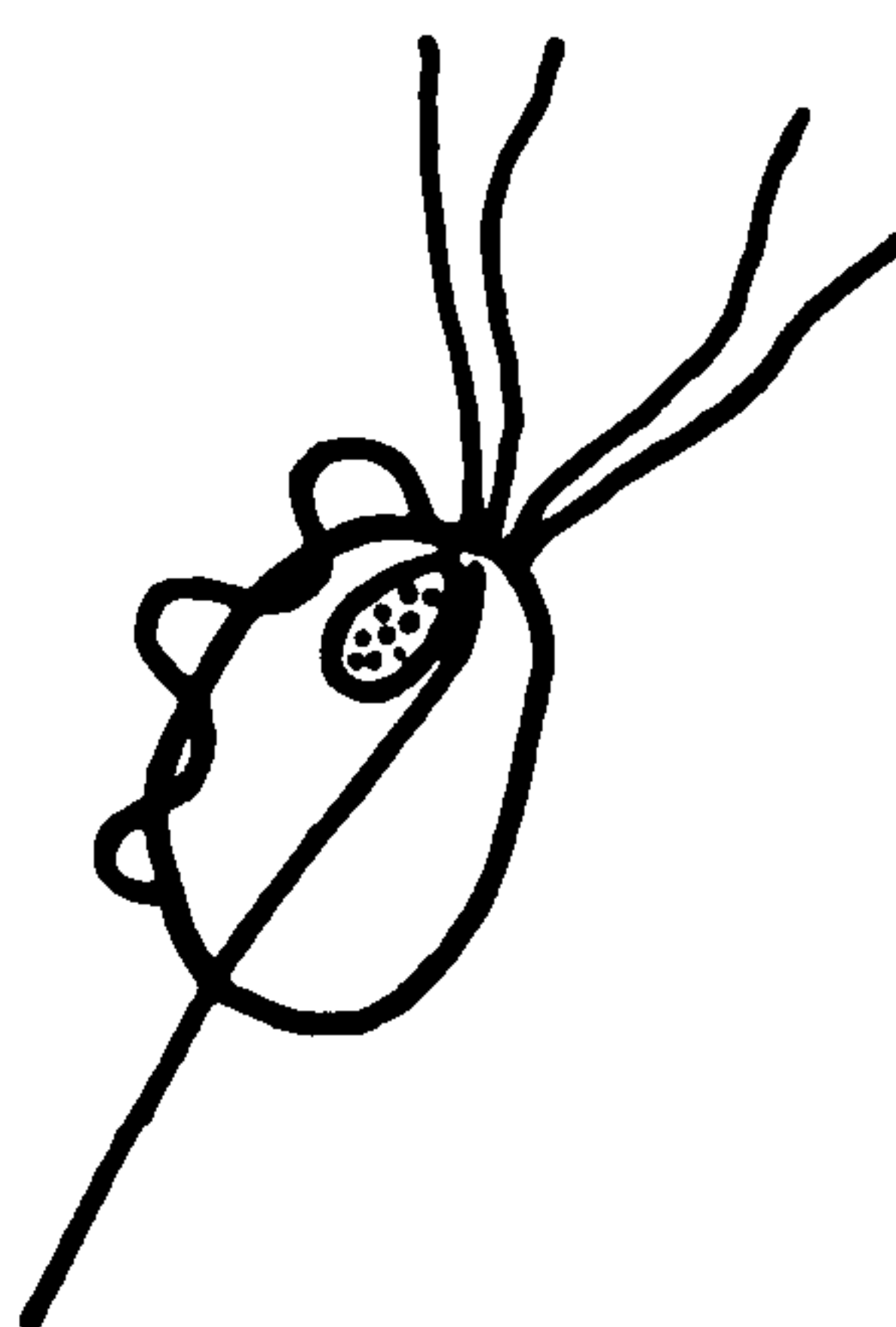


Trichomonas gallinae

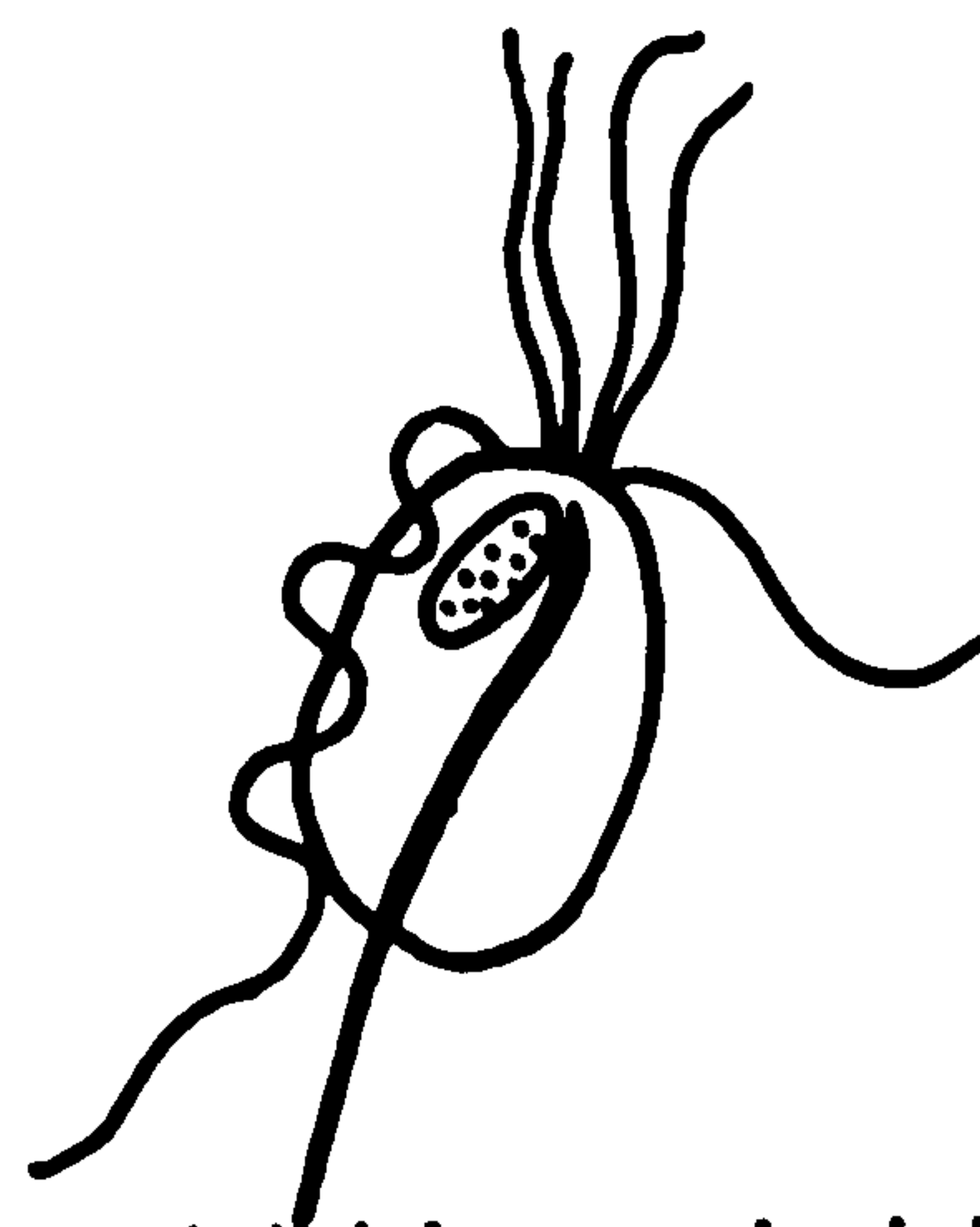
Trichomonas vaginalis



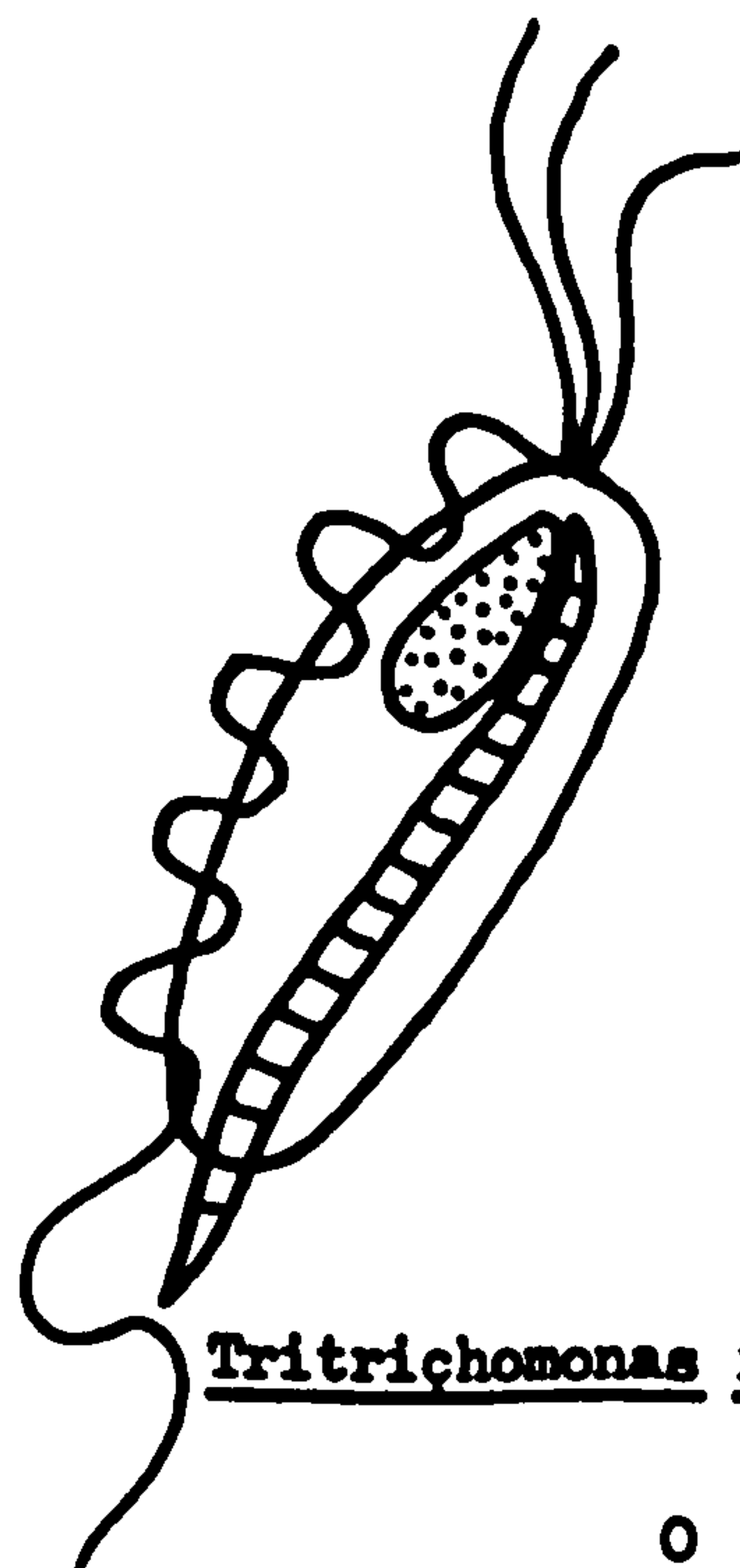
Tetratrichomonas gallinarum



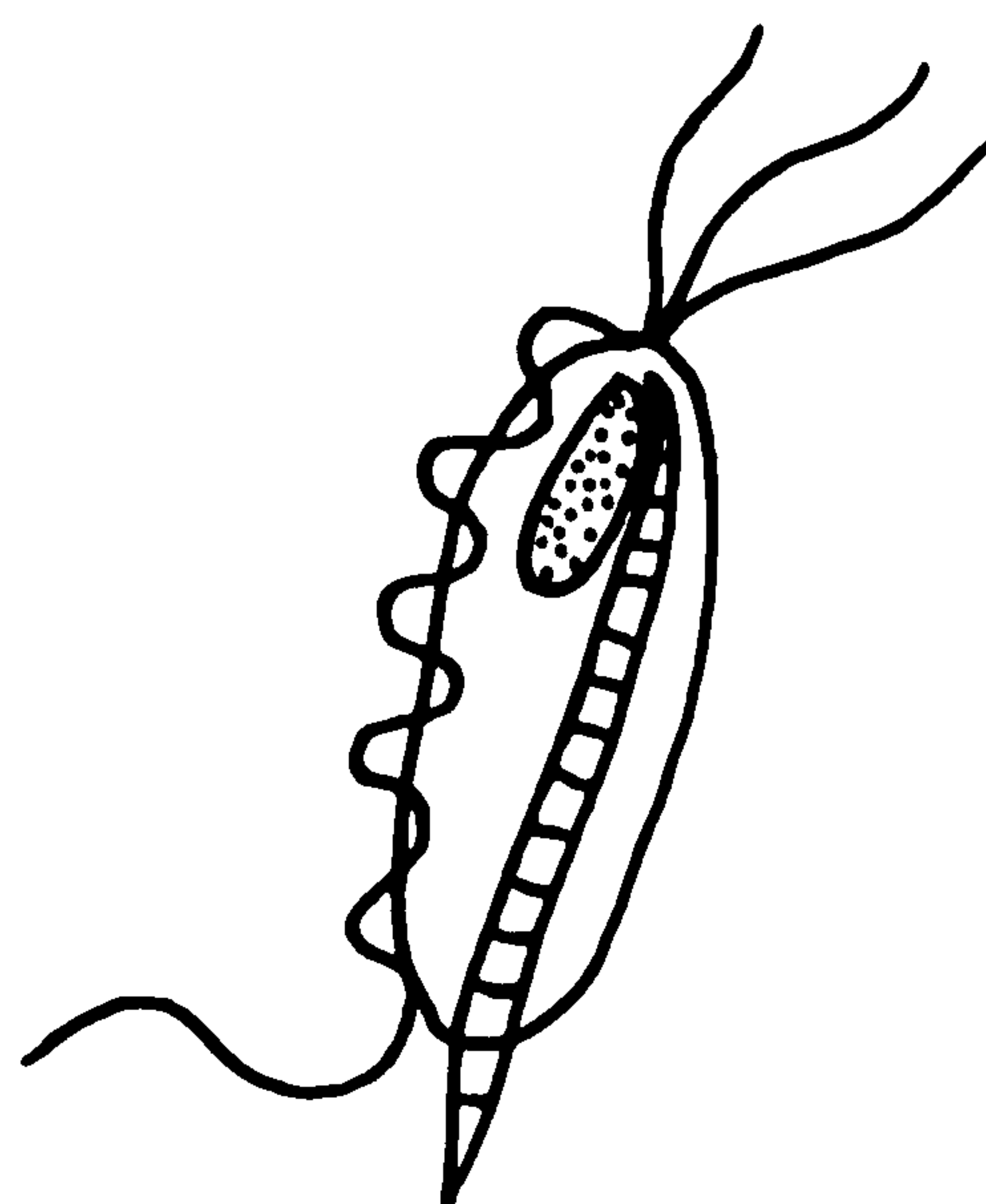
Trichomonas tenax



Pentatrichomonas hominis



Tritrichomonas foetus



Tritrichomonas suis

0 10 20  
μm

Fig. 1a

Fig. 1b. Sketch showing the main organelles of T. vaginalis. See list of abbreviations for explanation of symbols.

The organism is depicted as a longitudinal section of the body with the flagella and undulating membrane appearing in transverse section.



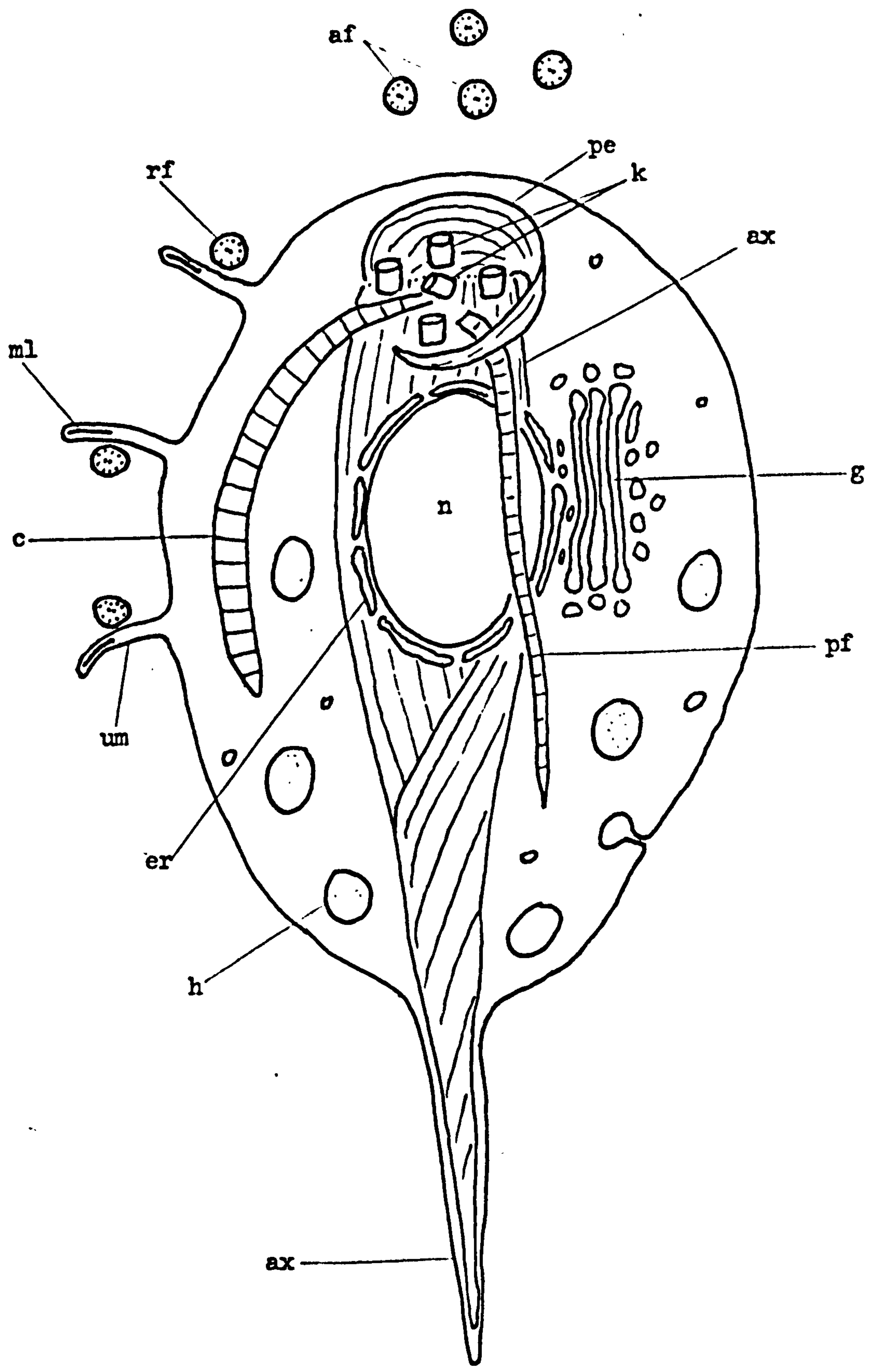


Figure 1b

2  $\mu$ m

Fig. 2. Photomicrograph of a 3 day old monolayer of rabbit kidney 13 epithelial cells. Giemsa stained preparation. Some binucleate (small arrows) and multinucleate (large arrow) cells are present. The cytoplasm of the cells contain numerous cytoplasmic vacuoles (va) up to 30  $\mu$ m in diameter.

Bar = 100  $\mu$ m

Fig. 3. Phase contrast micrograph of a RK 13 monolayer (ecm) 1 h post-infection with T. vaginalis showing an aggregate of about 100 organisms (tv) lying on the monolayer. Araldite embedded preparation. Two smaller clumps of T. vaginalis can be seen at the top and at bottom left of the figure. Note that only a few isolated T. vaginalis are seen between the aggregates (e.g. arrow).

Bar = 100  $\mu$ m.



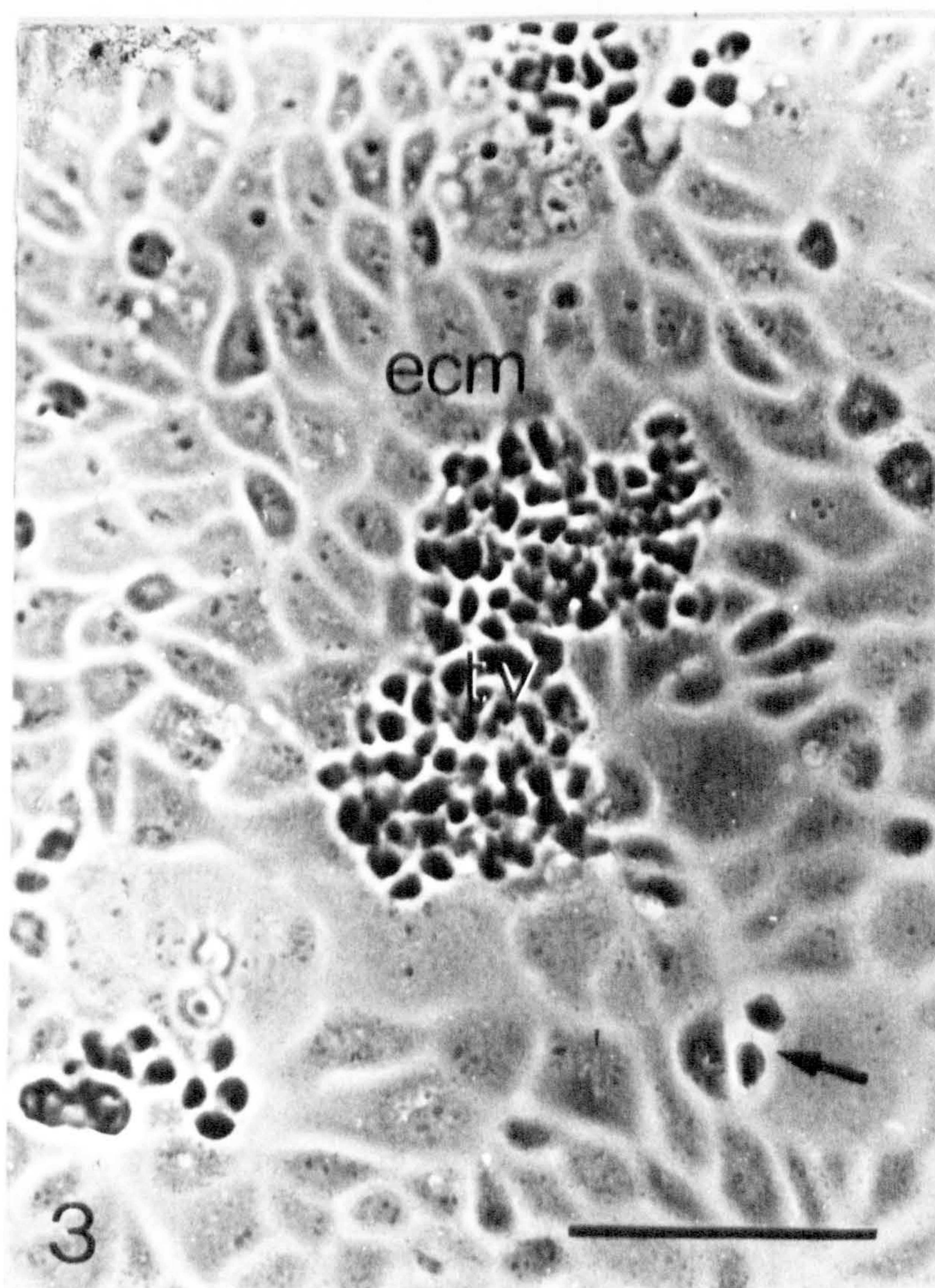
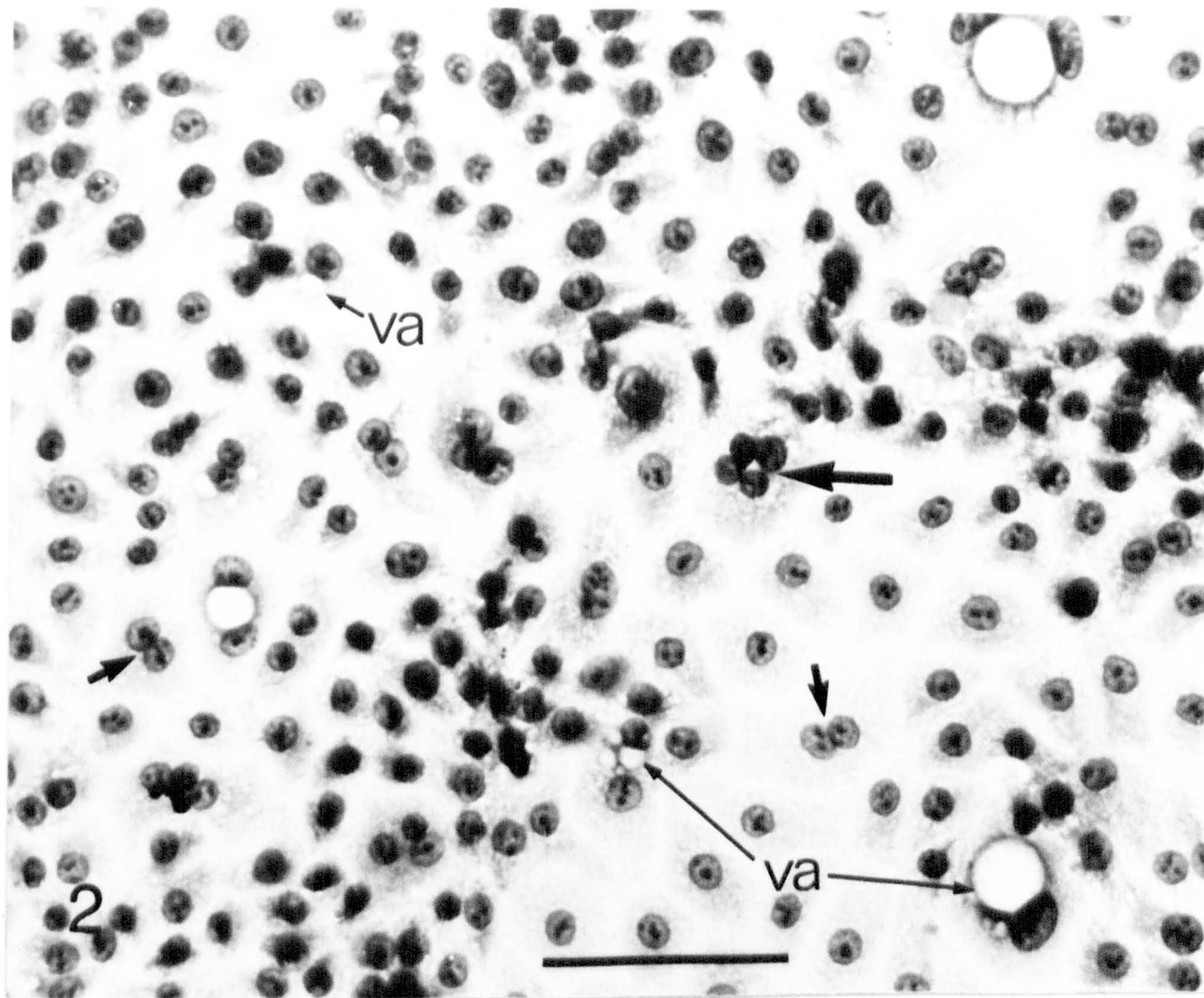




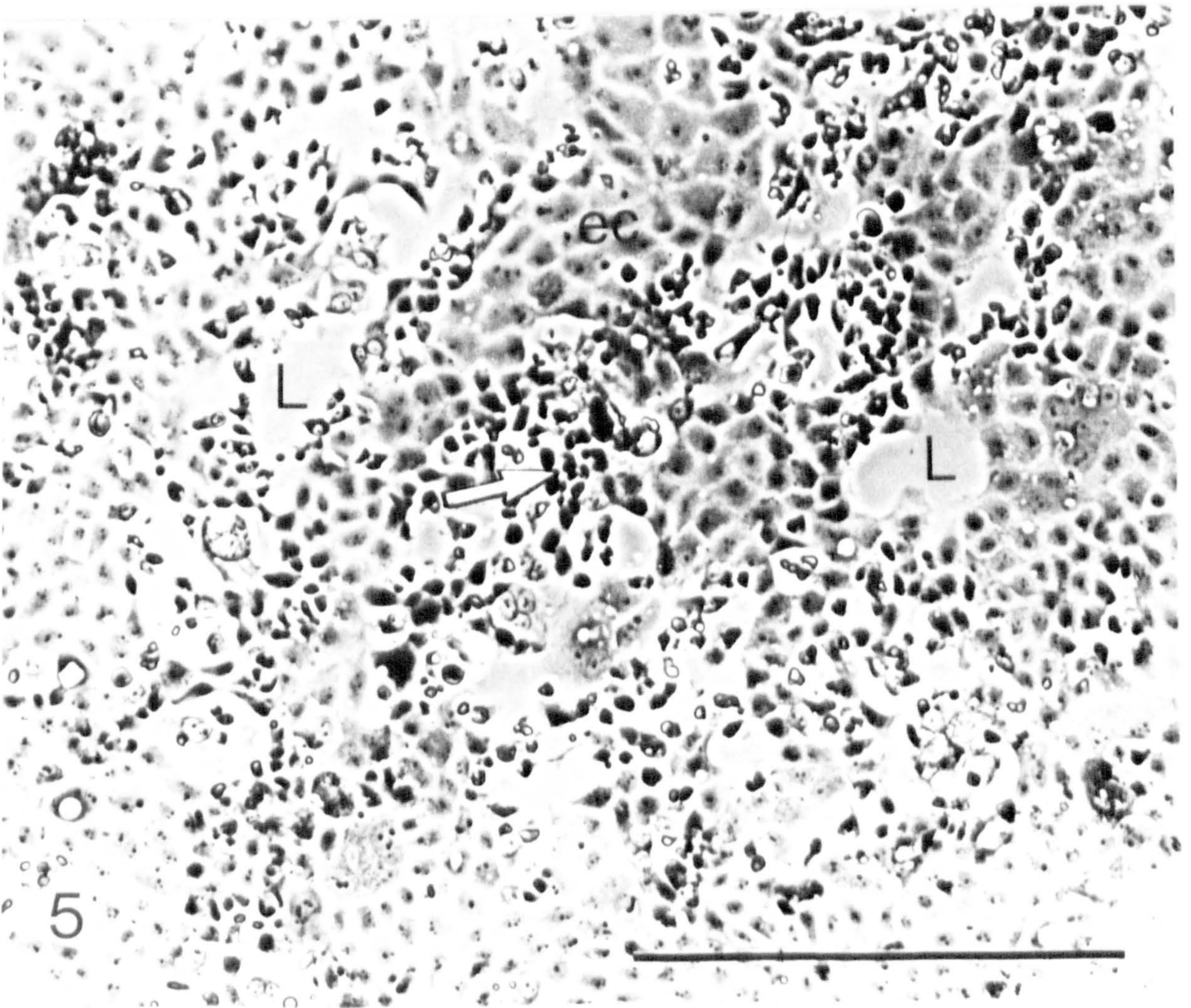
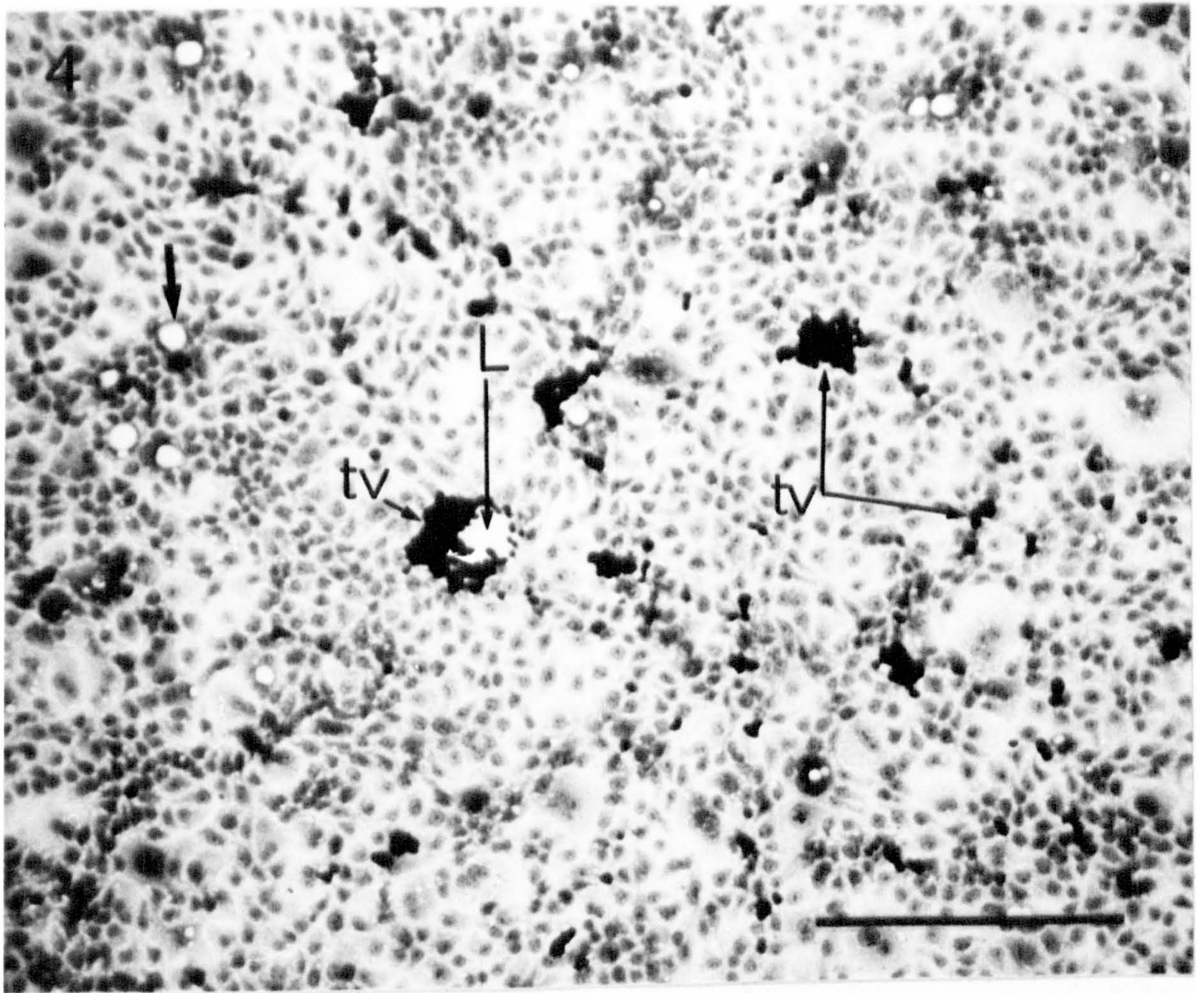
Fig. 4. RK 13 monolayer 2 h post-infection with T. vaginalis. Giemsa stained preparation. Darkly stained aggregates of trichomonads are adherent to the monolayer. A small cell-free area, or lesion (L), can be seen on the right of the largest aggregate. No lesions are present in the unparasitised areas of the monolayer. Some of the cells contain cytoplasmic vacuoles (arrow).

Bar = 250  $\mu$ m.

Fig. 5. PC micrograph of a RK 13 monolayer 6 h post-infection with T. vaginalis. Araldite embedded preparation. Numerous lesions are present in the monolayer. In the centre (arrow) a lesion is filled with trichomonads. Note the clear areas of substratum in the lesions (L).

Bar = 250  $\mu$ m.







Figs. 6 and 7. High power phase contrast micrographs of lesions (L) produced in RK 13 monolayers 6 h post-infection with T. vaginalis. Araldite embedded preparations.

Bars = 50  $\mu$ m

Fig. 6. Two lesions separated by a large aggregate of T. vaginalis. Note that few T. vaginalis are lying in the centres of the lesions; many amoeboid organisms are lying either partly (small arrows) or completely (open arrow) beneath the epithelial cells which line the lesions. The nuclei of damaged or lysed cells are often pyknotic with an increased density in phase contrast optics (large arrow). The monolayer of cells (ecm) surrounding the lesions appears undamaged, but some cells contain large vacuoles (va).

Fig. 7. Many T. vaginalis are palisaded against the epithelial cells lining the lesions. The nucleus of a lysed cell is arrowed. Note the retraction fibres (rt) which extend across the lesion from cells at the edge.



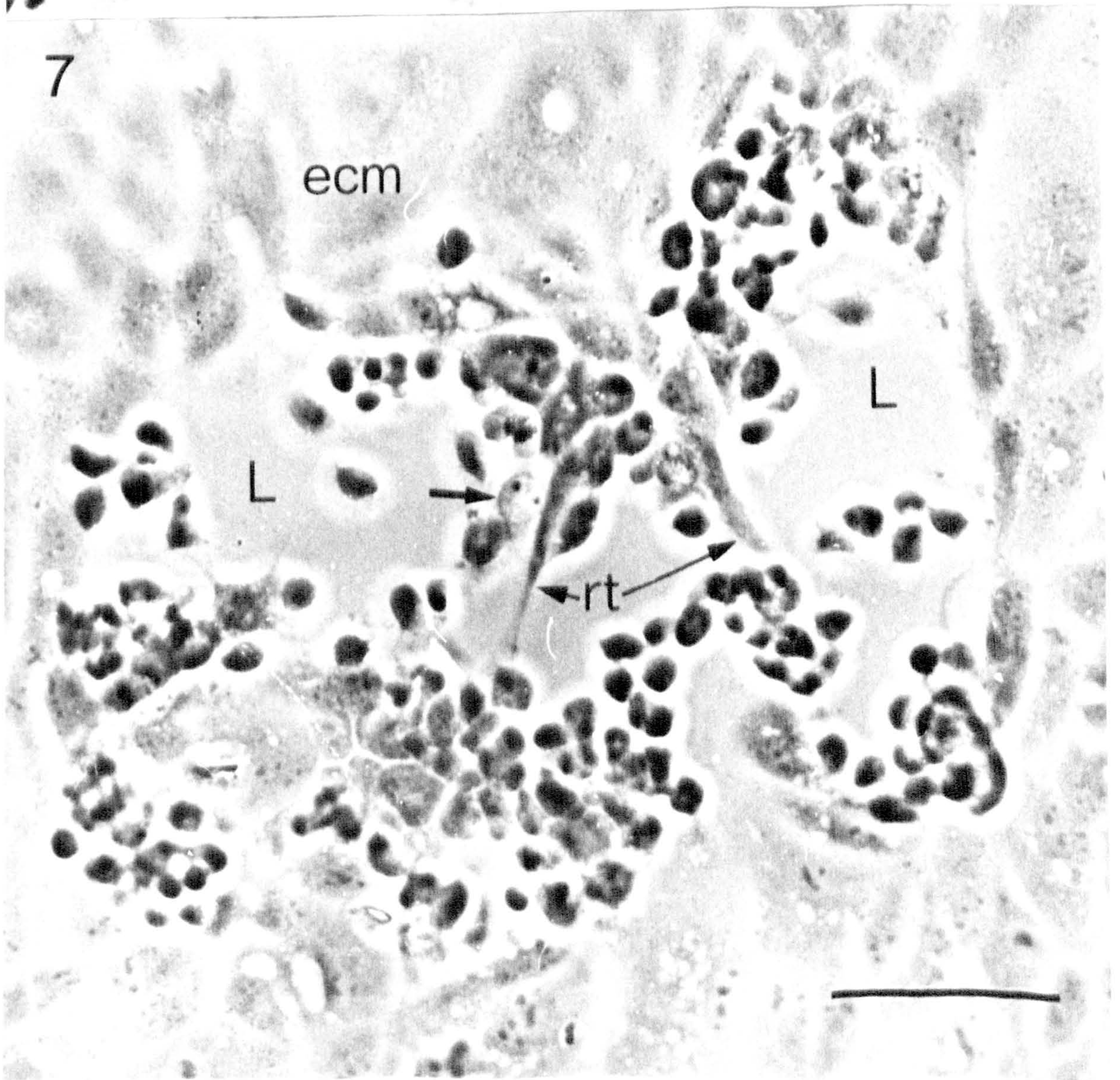
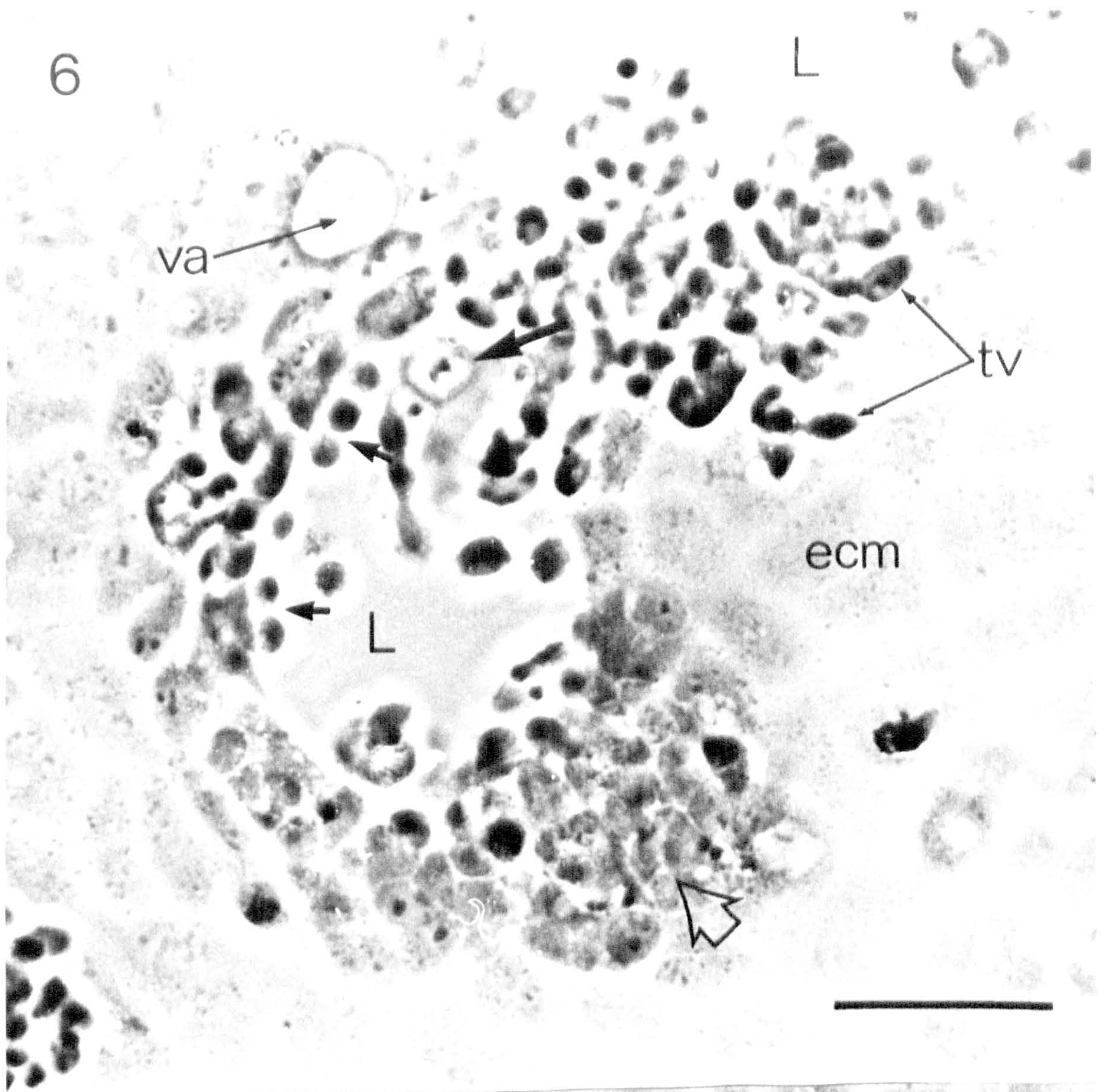




Fig. 8. RK 13 monolayer 10 h post-infection with T. vaginalis. Giemsa stained preparation. A large lesion, approximately 860  $\mu\text{m}$  by 470  $\mu\text{m}$  is present in the monolayer. There are many darkly stained T. vaginalis lining the edge of the lesion. Note that the unparasitised area of the monolayer at lower right appears undamaged and is free of lesions.

Bar = 100  $\mu\text{m}$ .

Fig. 9. RK 13 monolayer 36 h post-infection with T. vaginalis. Giemsa stained preparation. Most of the monolayer has been destroyed leaving small islands of cells to which many T. vaginalis are adherent. Many organisms are adhering to the exposed substratum. An unusually large trichomonad is arrowed.

Bar = 50  $\mu\text{m}$ .



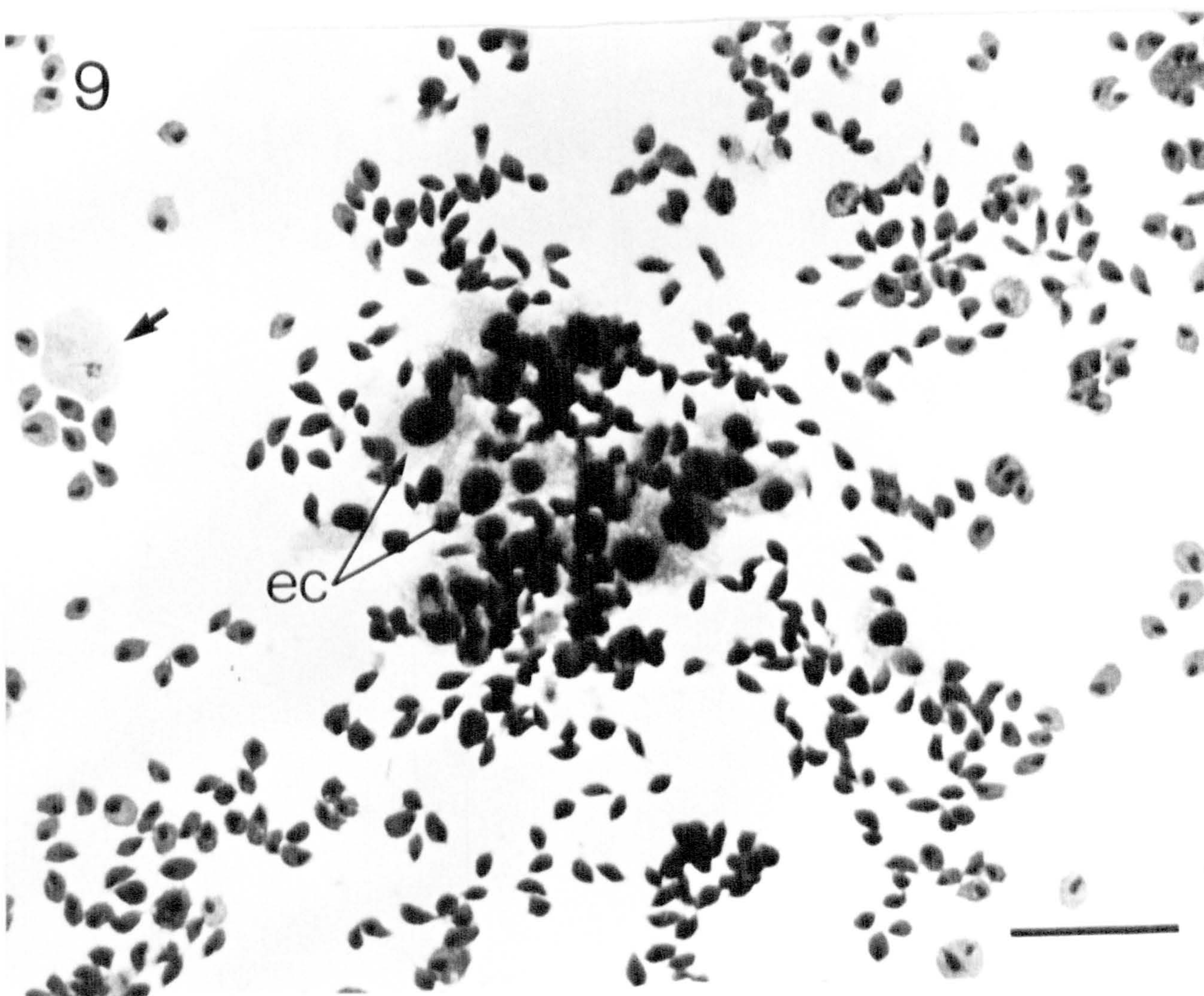
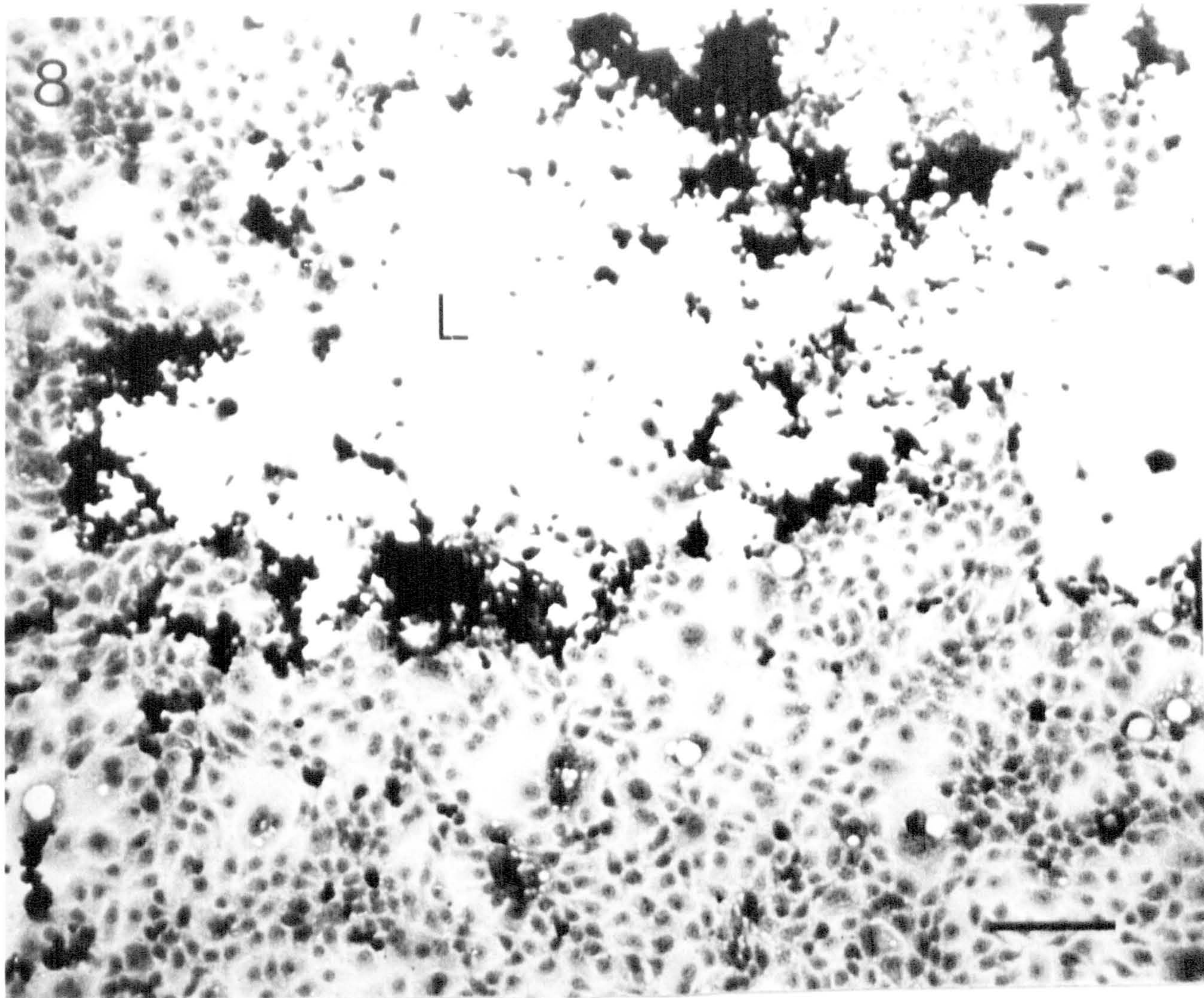




Fig. 10. Scanning electron micrograph of an amoeboid T. vaginalis adhering to a glass substratum, showing the main surface features of the organism. The anterior flagella (af) emerge from the anterior pole. This is probably an early dividing form of T. vaginalis since two undulating membranes (um) are present. At the rear of the organism the axostyle (ax) projects for 6  $\mu\text{m}$ ; the axostyle is 0.5  $\mu\text{m}$  in diameter at its base but tapers to a fine point. Note the flat pseudopodium (p) at the rear and on the left side of the body, and the short filopodium (fi) on the right side.

Bar = 5  $\mu\text{m}$ .

Fig. 11. Scanning electron micrograph of an amoeboid T. vaginalis adhering to the glass substratum in the centre of a lesion in an epithelial cell monolayer. A single broad and flat pseudopodium (p) has been protruded from the posterior pole of the trichomonad; the anterior half of the organism from which the flagella (af) and undulating membrane (um) project, is rounded and lifted off the substratum. Note the straight filopodia (fi) which project from the sides of the pseudopodium and are attached to the substratum at their distal ends (the filopodia have fractured during the critical point drying process).

Bar = 2  $\mu\text{m}$ .



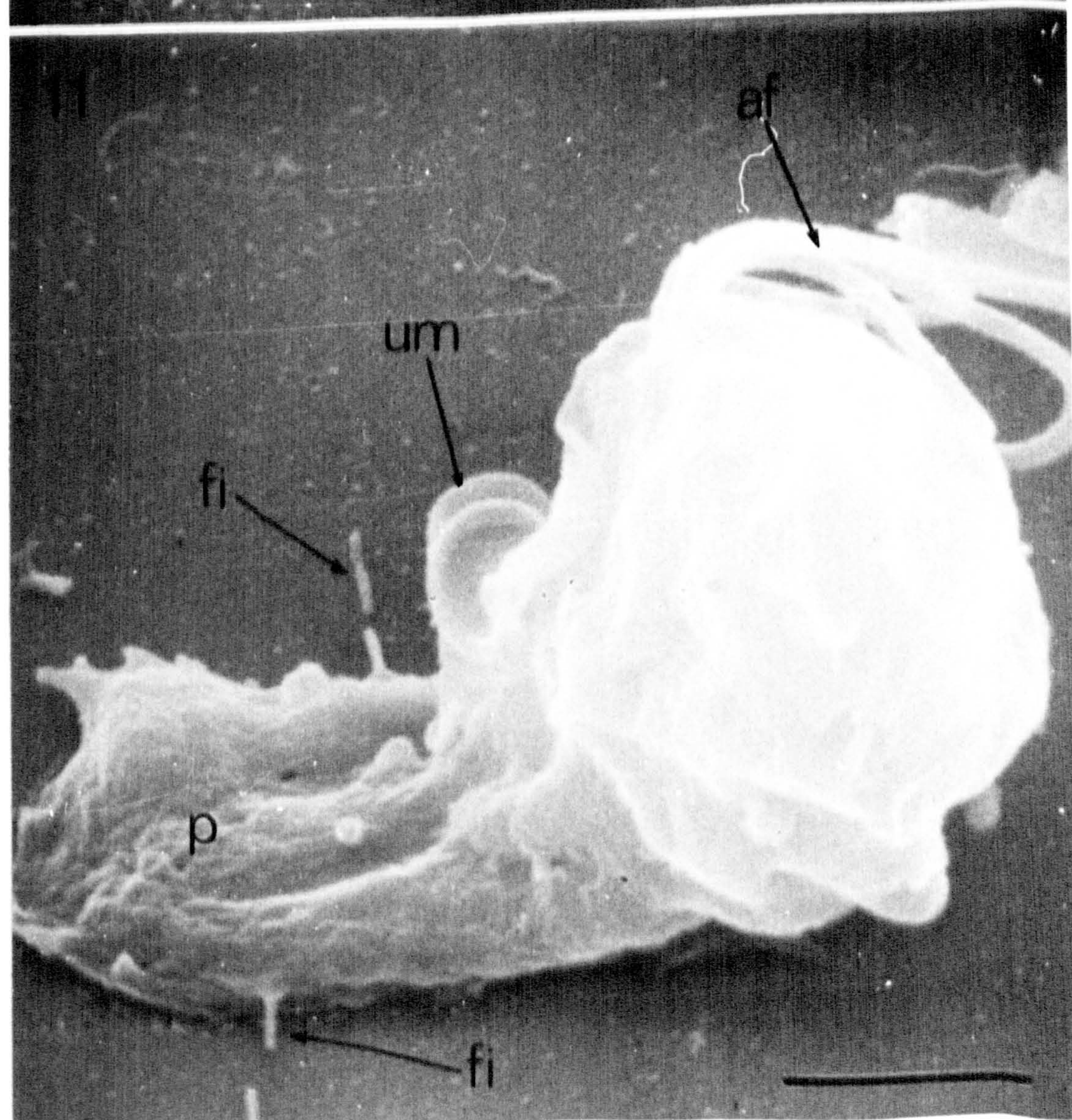
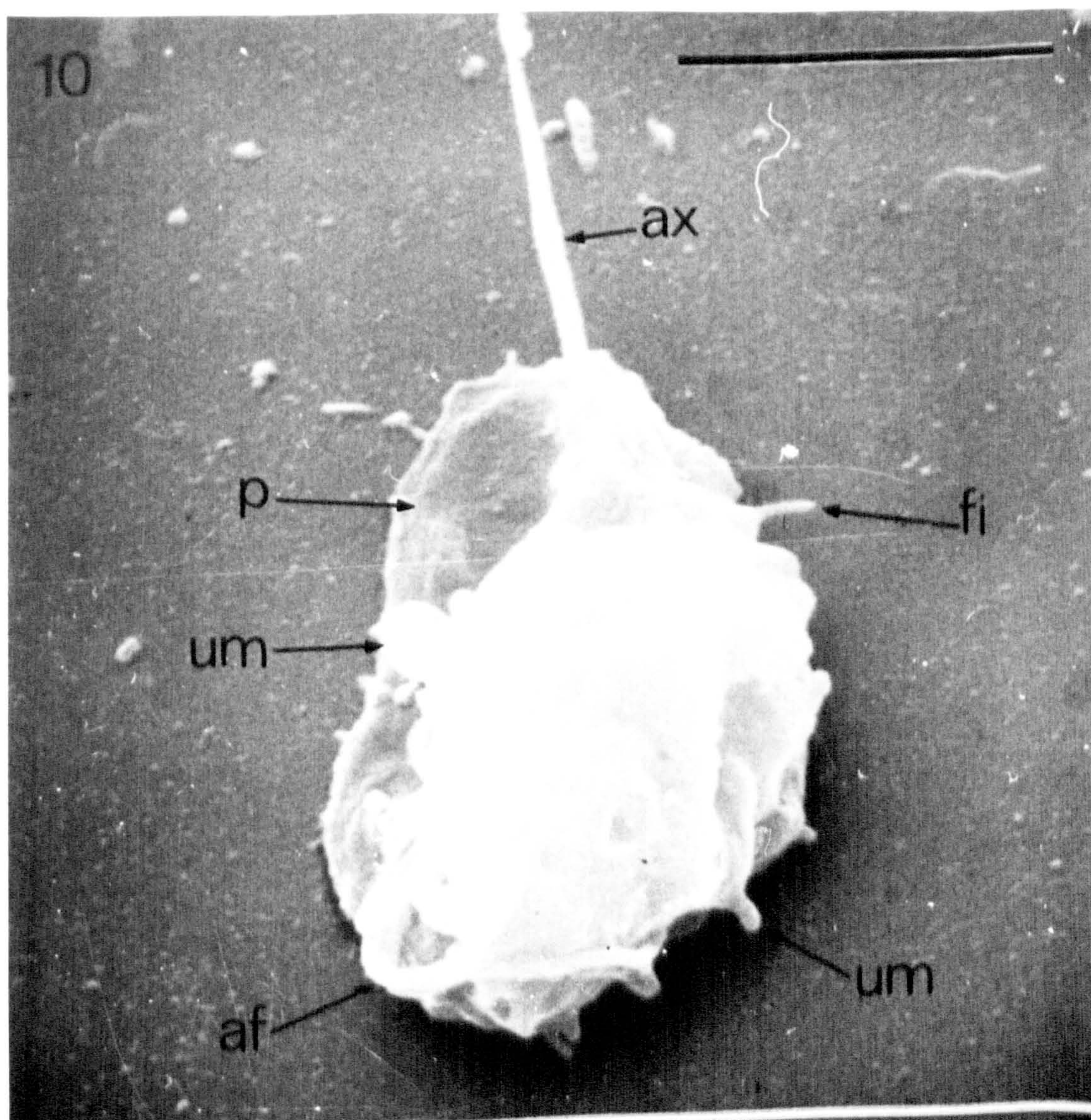




Fig. 12. Amoeboid T. vaginalis adhering to a glass substratum. The upper trichomonad is polarised along its anterior-posterior axis; the flagella (af) can be seen projecting from the anterior pole. Note how the trichomonad appears to be stretched out between adhesions to the substratum at the poles of the body; the central part of the body is lifted away from the substratum and its side describes a catenary curve (arrow). The spherical object next to the lower trichomonad originates from the epithelial cell monolayer.

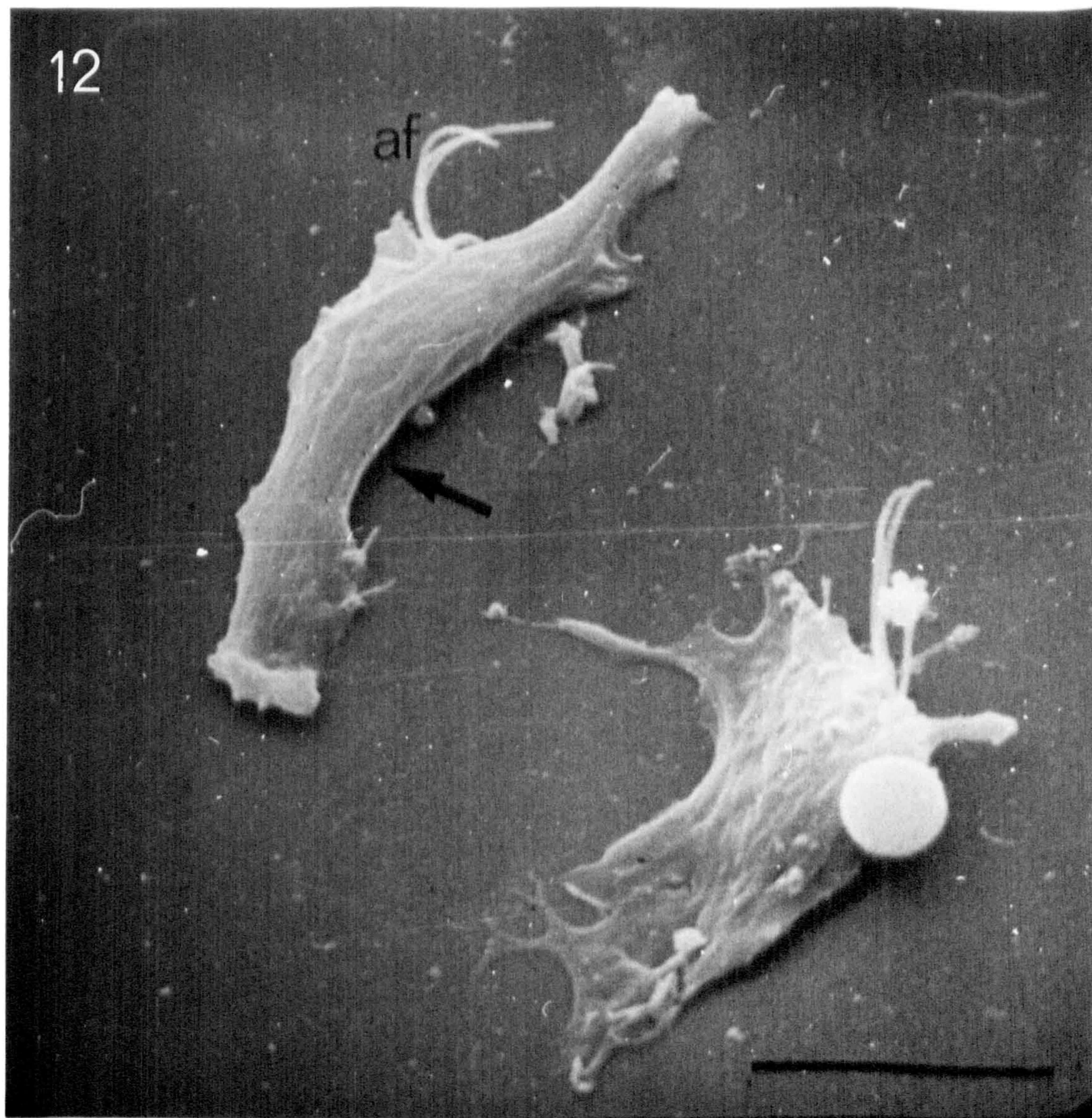
Bar = 10  $\mu$ m.

Fig. 13. A group of eight T. vaginalis adhering to the exposed substratum in the centre of a lesion. Note the broad flat pseudopodia (arrows) which lie beneath the rounded central parts of the bodies of the organisms, forming a confluent sheet of pseudopodia.

Bar = 10  $\mu$ m.



12



13

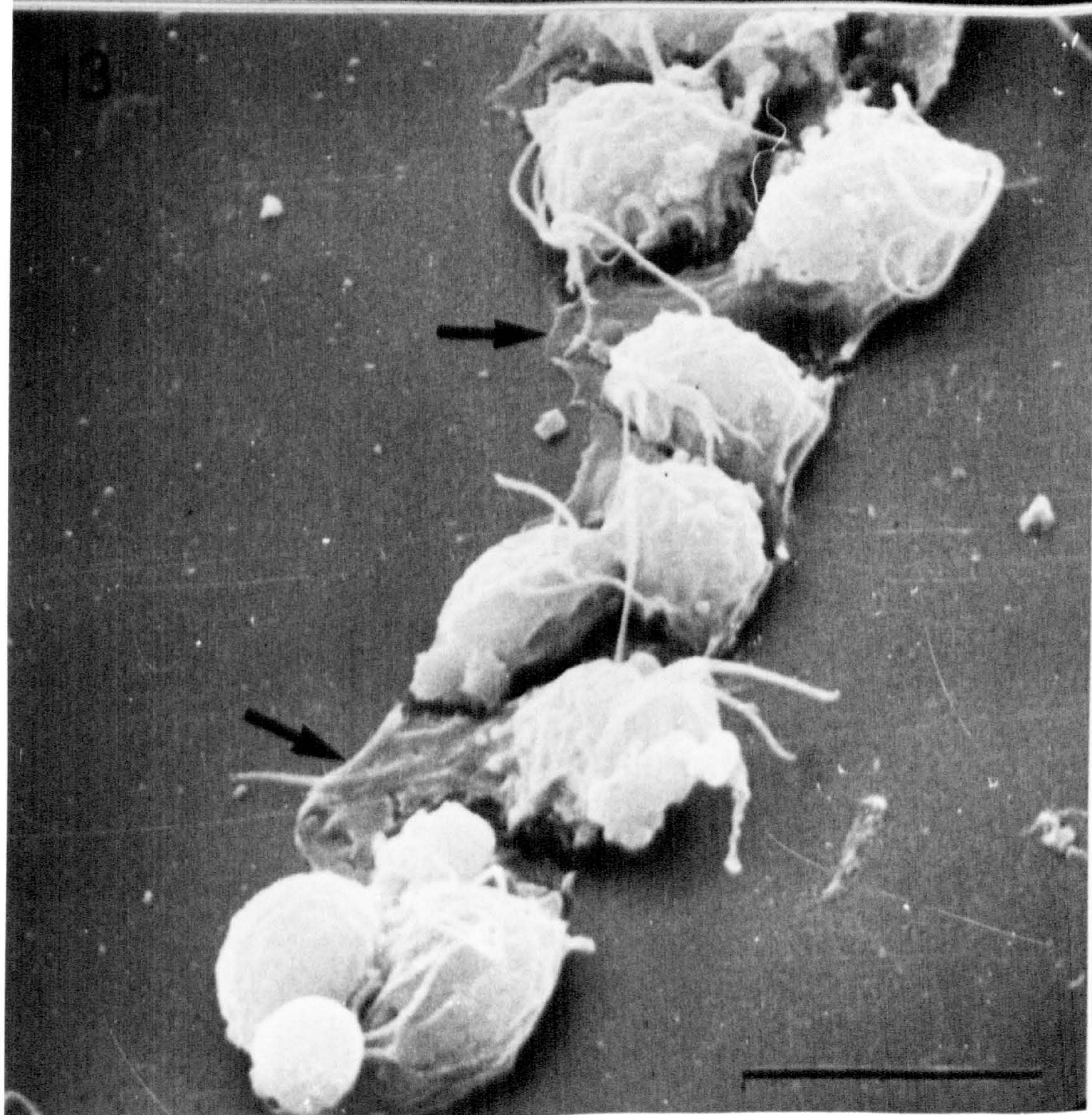




Fig. 14. Anterior pole of T. vaginalis showing the four anterior flagella and the undulating membrane (um). The um is a thin fold of cytoplasm to which the recurrent flagellum (rf) is attached at a distance of 0.5  $\mu$ m from its free edge. The um and rf pass backwards together and terminate half way down the body of T. vaginalis (arrow). The parasite lies in close contact with an epithelial cell (ec).

Bar = 2  $\mu$ m.

Fig. 15. High magnification view of the anterior pole of T. vaginalis showing the flagellar pocket (fp) from which the four anterior flagella, the recurrent flagellum and the undulating membrane emerge.

Bar = 1  $\mu$ m.



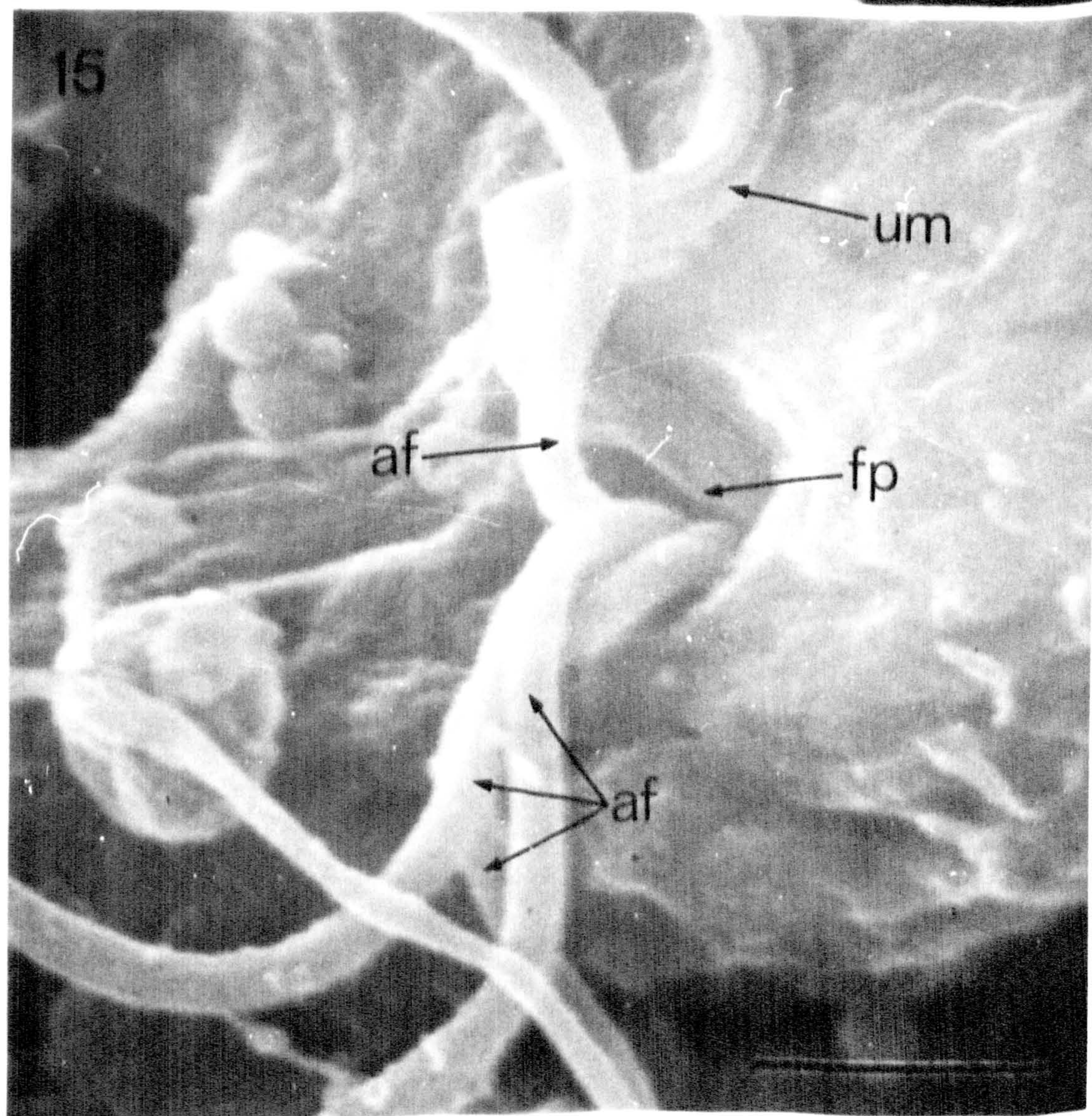
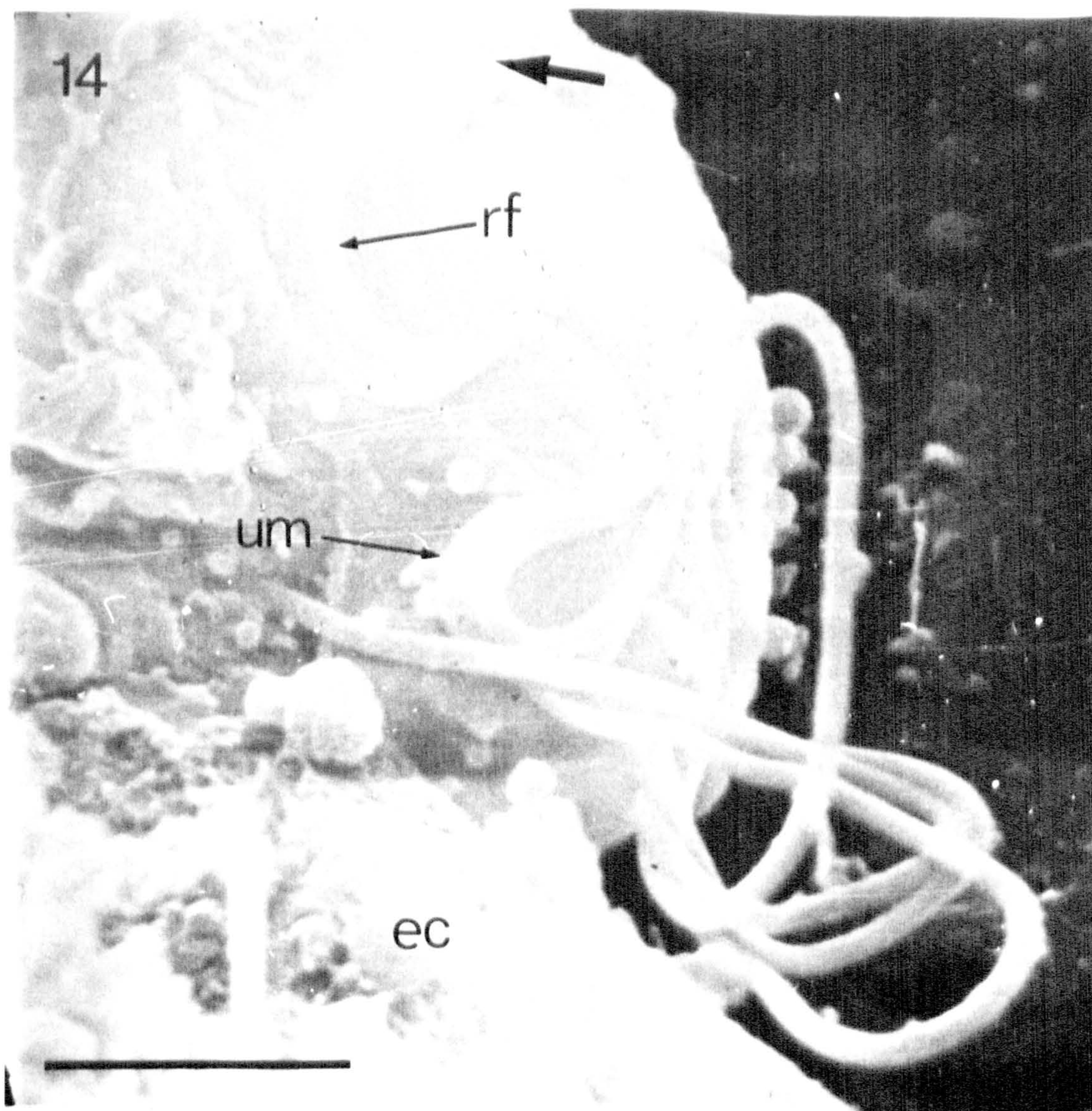




Fig. 16. High magnification view of the anterior flagella (af), undulating membrane (um) and recurrent flagellum (rf) of T. vaginalis.

Bar = 1  $\mu$ m.

Fig. 17. A late dividing form of T. vaginalis. Two daughter trichomonads are seen separated by a cleavage furrow (between black arrows). Both daughters have undulating membranes which pass backwards on the upper surface and terminate halfway along the body. Note that the undulating membrane and recurrent flagellum terminate together (open arrow).

Bar = 2  $\mu$ m.



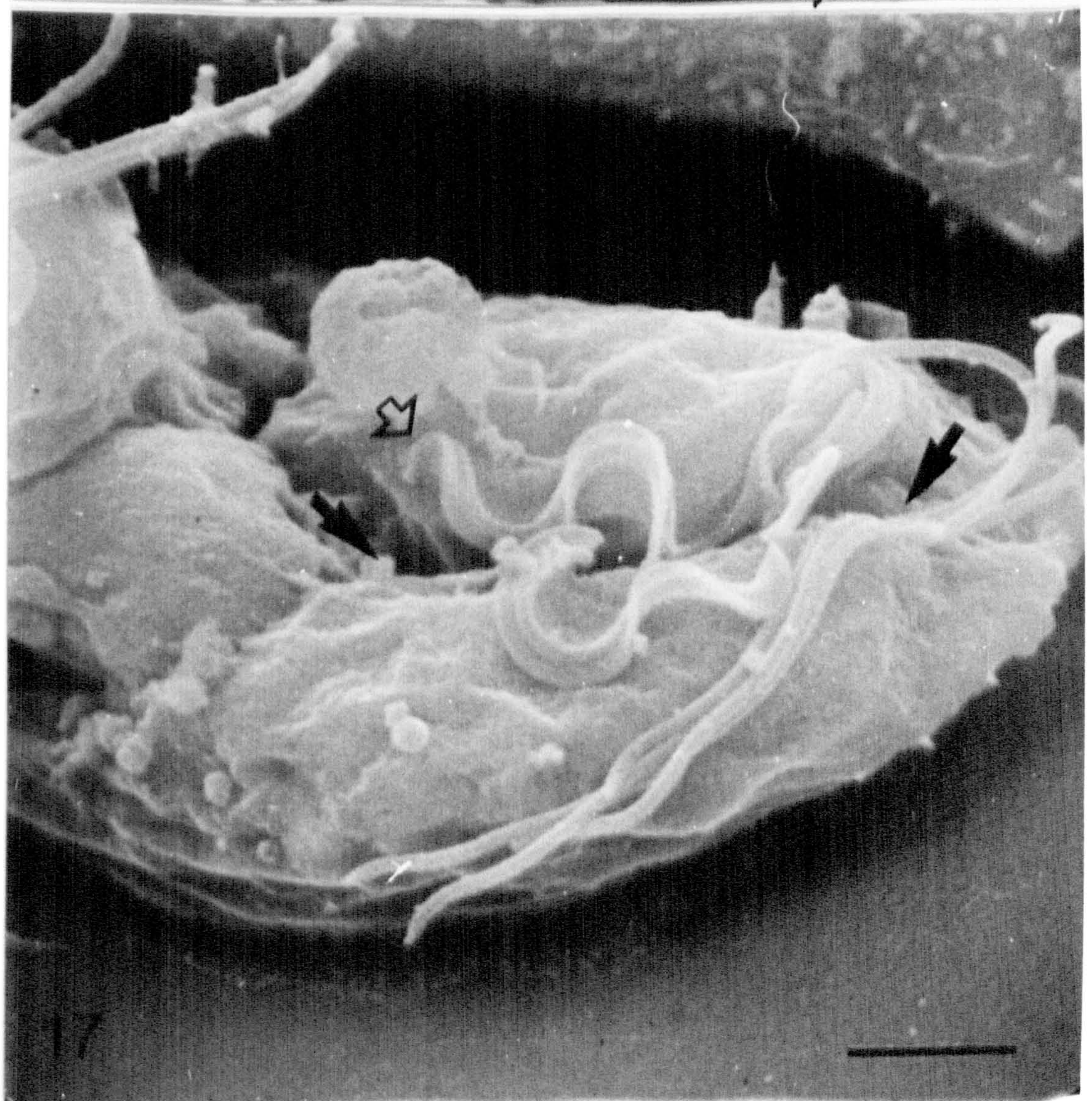
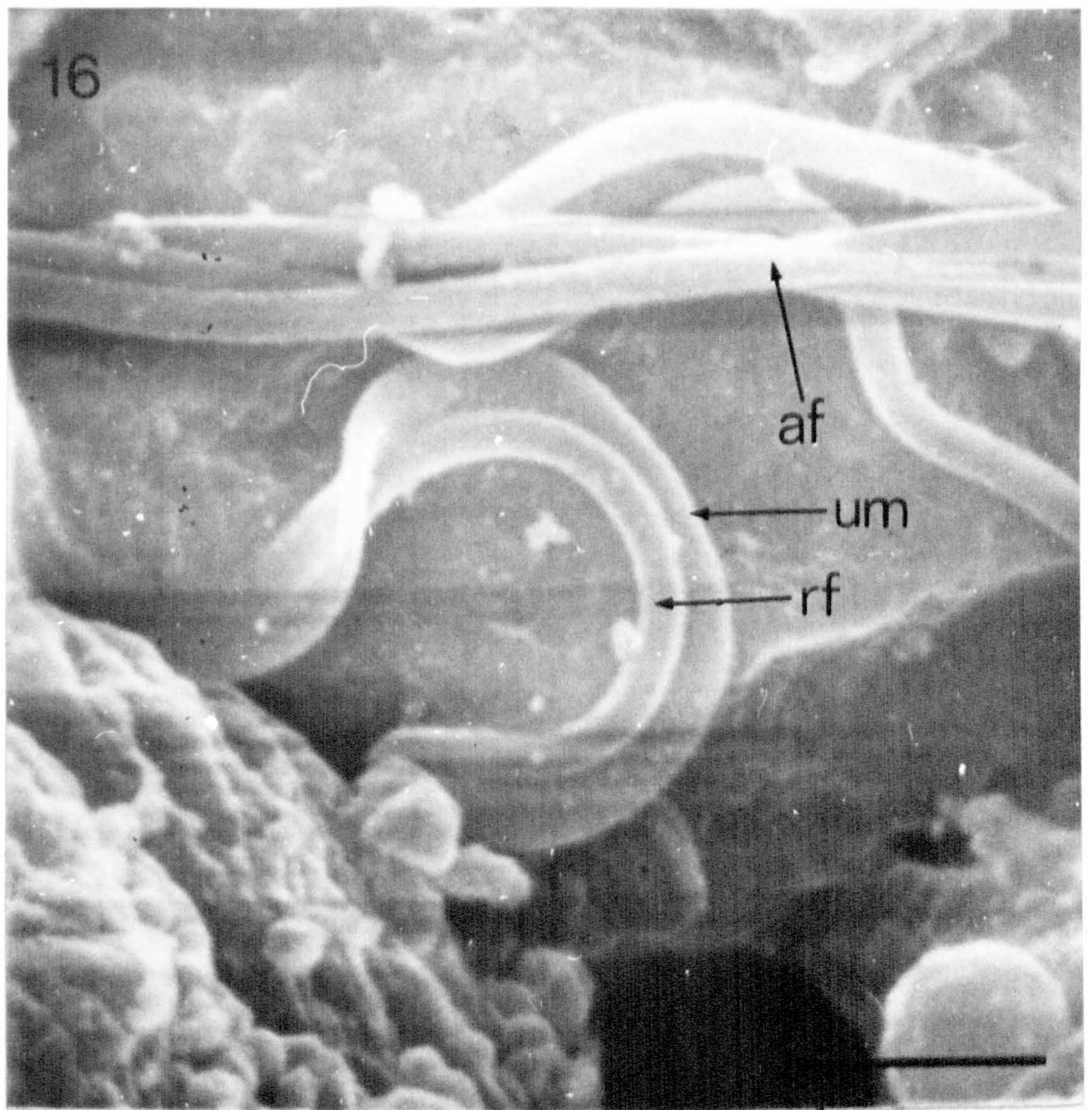




Fig. 18. Abnormal dividing form of T. vaginalis. The body is disc-shaped, 25  $\mu\text{m}$  in diameter, and has at least 5 separate sets of flagella. There is a long pseudopodium on one side of the body.

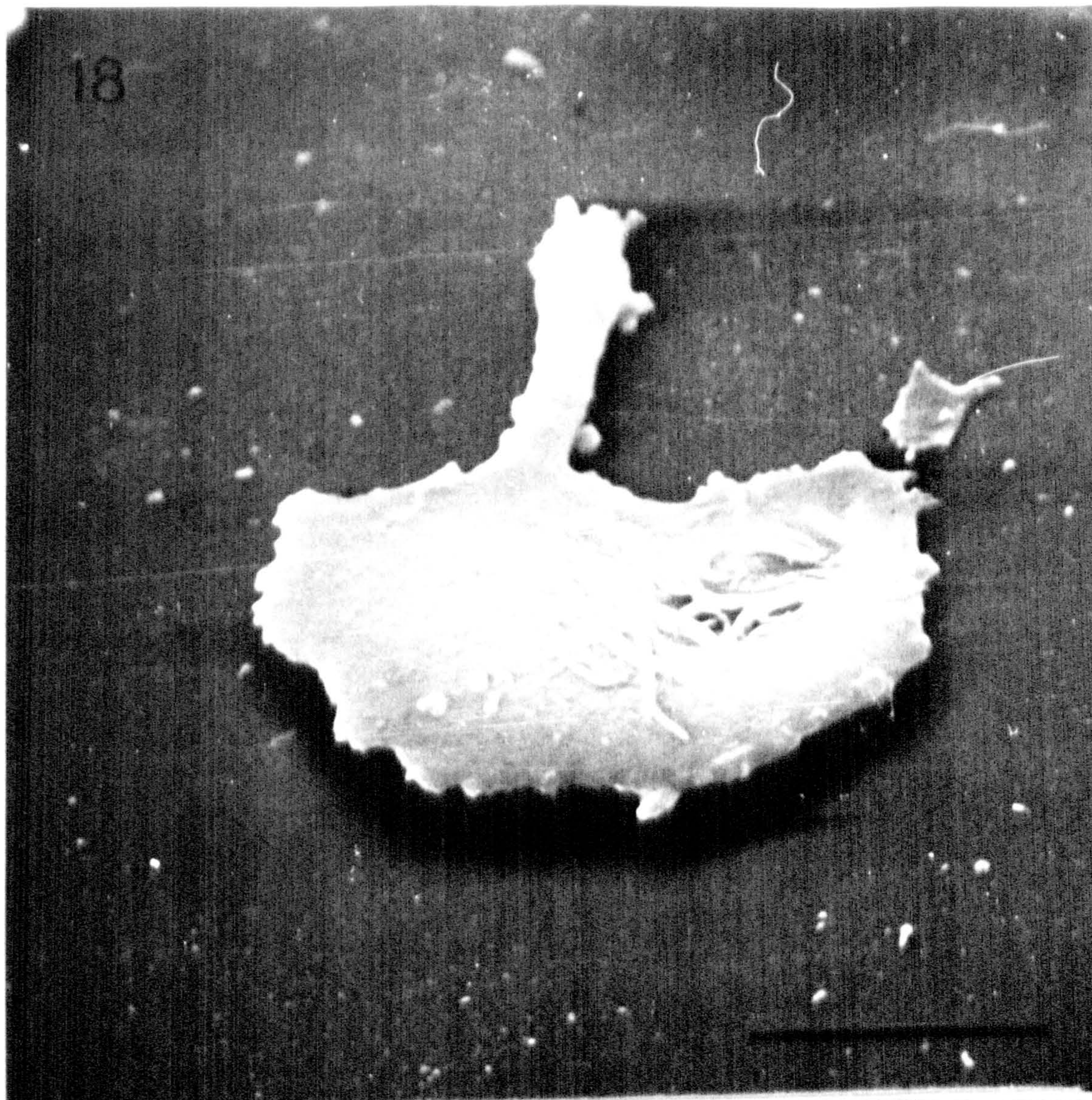
Bar = 10  $\mu\text{m}$ .

Fig. 19. Scanning electron micrograph of a 3 day old monolayer of RK 13 epithelial cells. The monolayer consists of a sheet of polygonal-shaped cells 20 to 50  $\mu\text{m}$  in diameter. The spaces between the cells are an artefact of the critical point drying process.

Bar = 100  $\mu\text{m}$ .



18



19

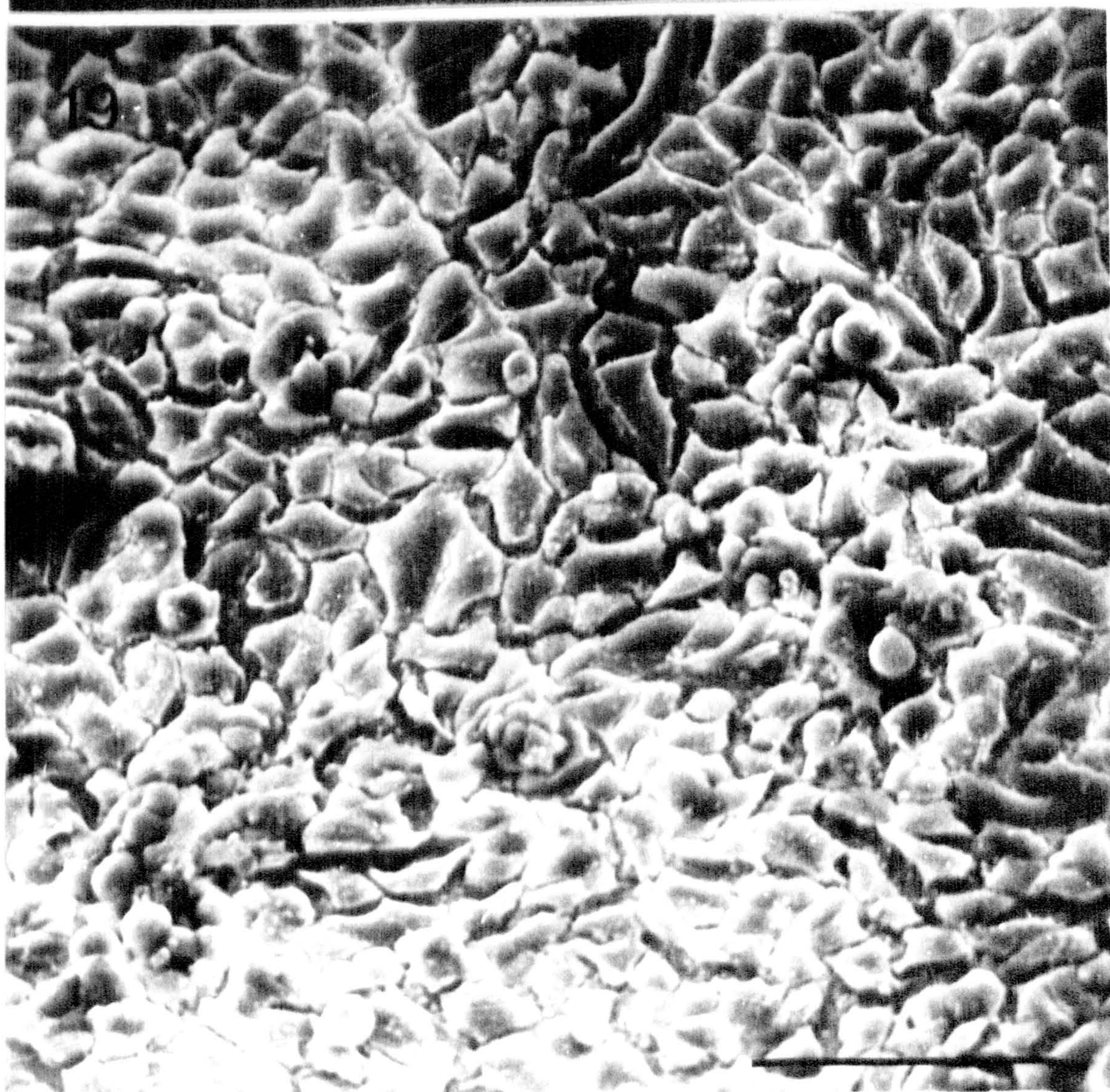




Fig. 20. A lesion in a monolayer of epithelial cells infected for 6 h with T. vaginalis. The lesion is 400  $\mu\text{m}$  at its widest diameter; only a few epithelial cells are present in the centre of the lesion, which is occupied mainly by T. vaginalis (small arrows). An aggregate of T. vaginalis lies on the monolayer at the top left of the figure. (large arrow).

Bar = 100  $\mu\text{m}$ .

Fig. 21. Detail of Fig. 20 showing the centre of the lesion. Note the cell free spaces in the lesion which are traversed by retraction fibres extending from the epithelial cells (arrows). Some trichomonads (tv) are adhering to the upper surfaces of the cells.

Bar = 50  $\mu\text{m}$ .



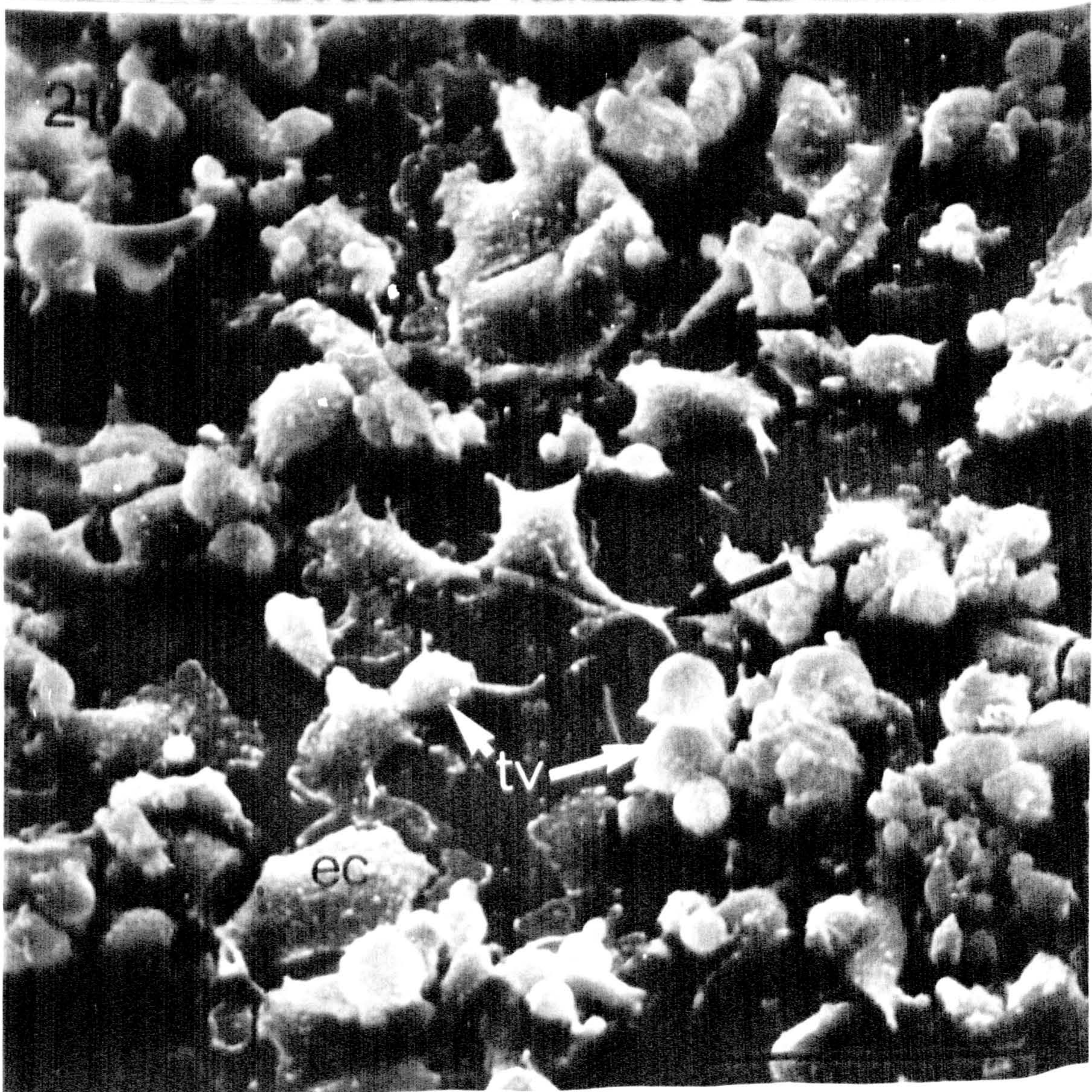
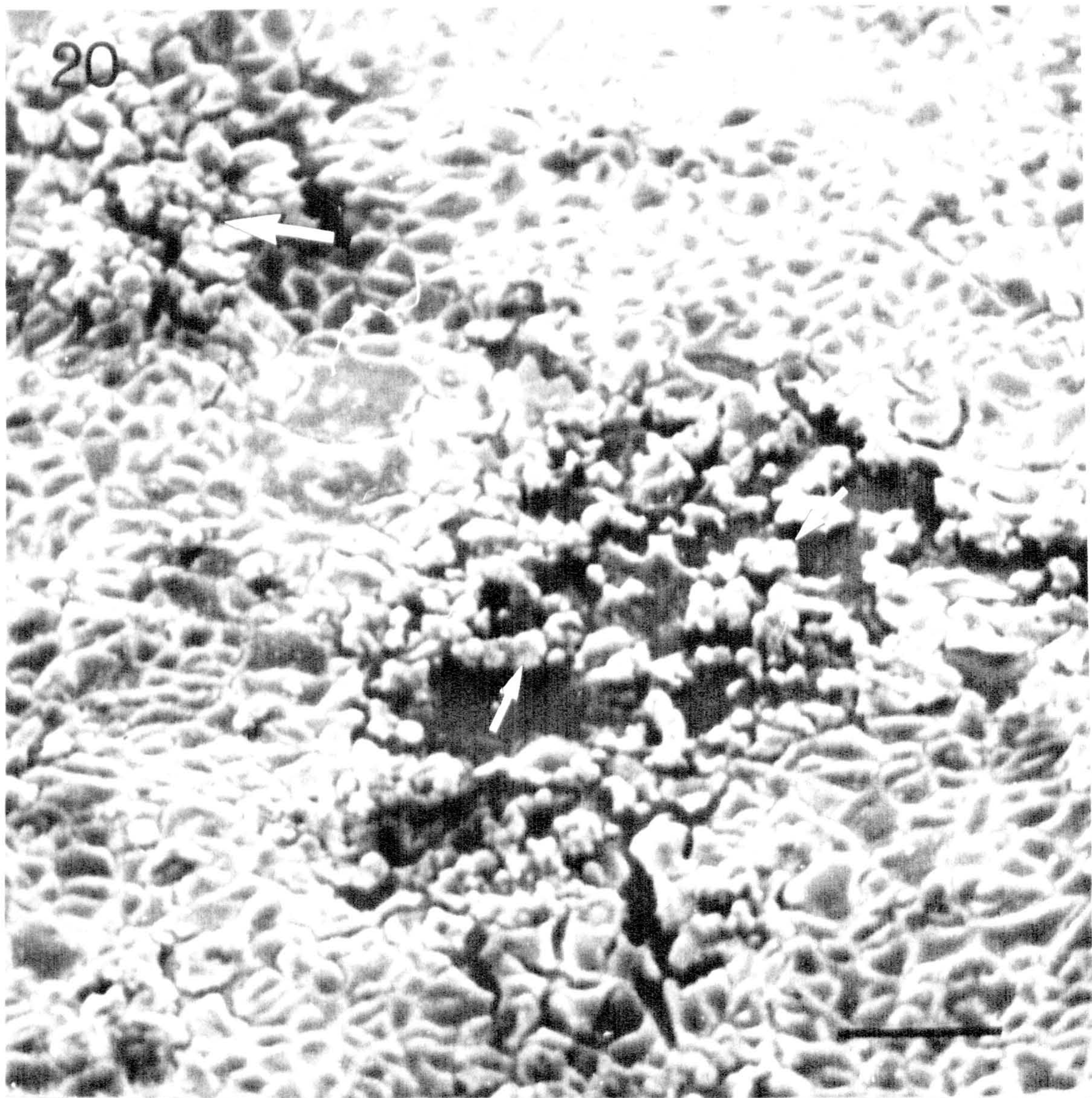




Fig. 22. A small lesion (L) in an epithelial cell monolayer infected for 6 h with T. vaginalis. Parasites are adhering to the cells at the edge of the lesion. Cells at one side of the lesion have been lifted away from the substratum (arrow).

Bar = 20  $\mu$ m.

Fig. 23. T. vaginalis adhering to epithelial cells (ec) at the edge of a lesion. Some of the cells have rounded up losing their normal flattened morphology. Note the large lamella projecting into the cell free areas of the lesion from a cell at the top of the figure (1a).

Bar = 20  $\mu$ m.



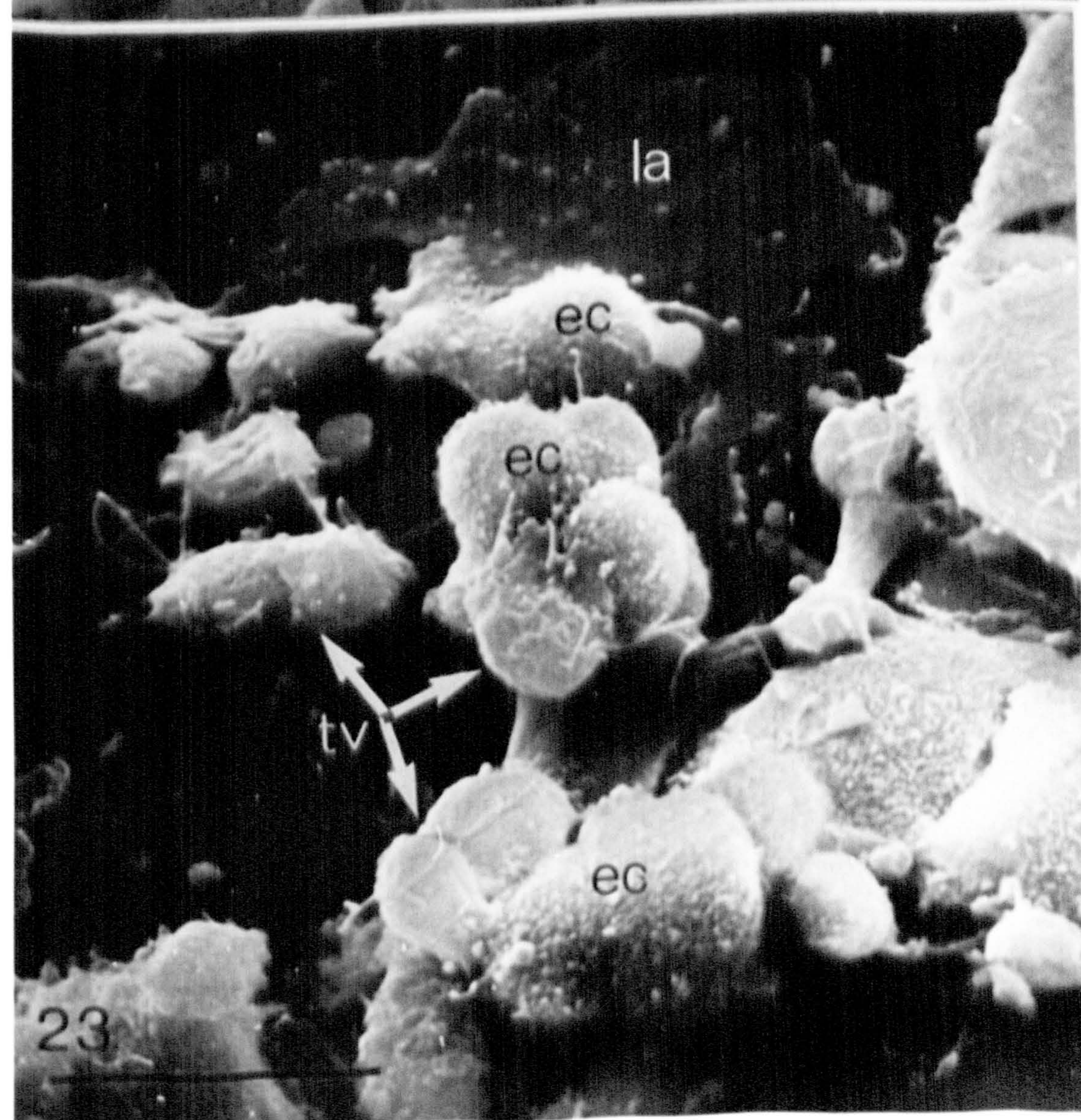
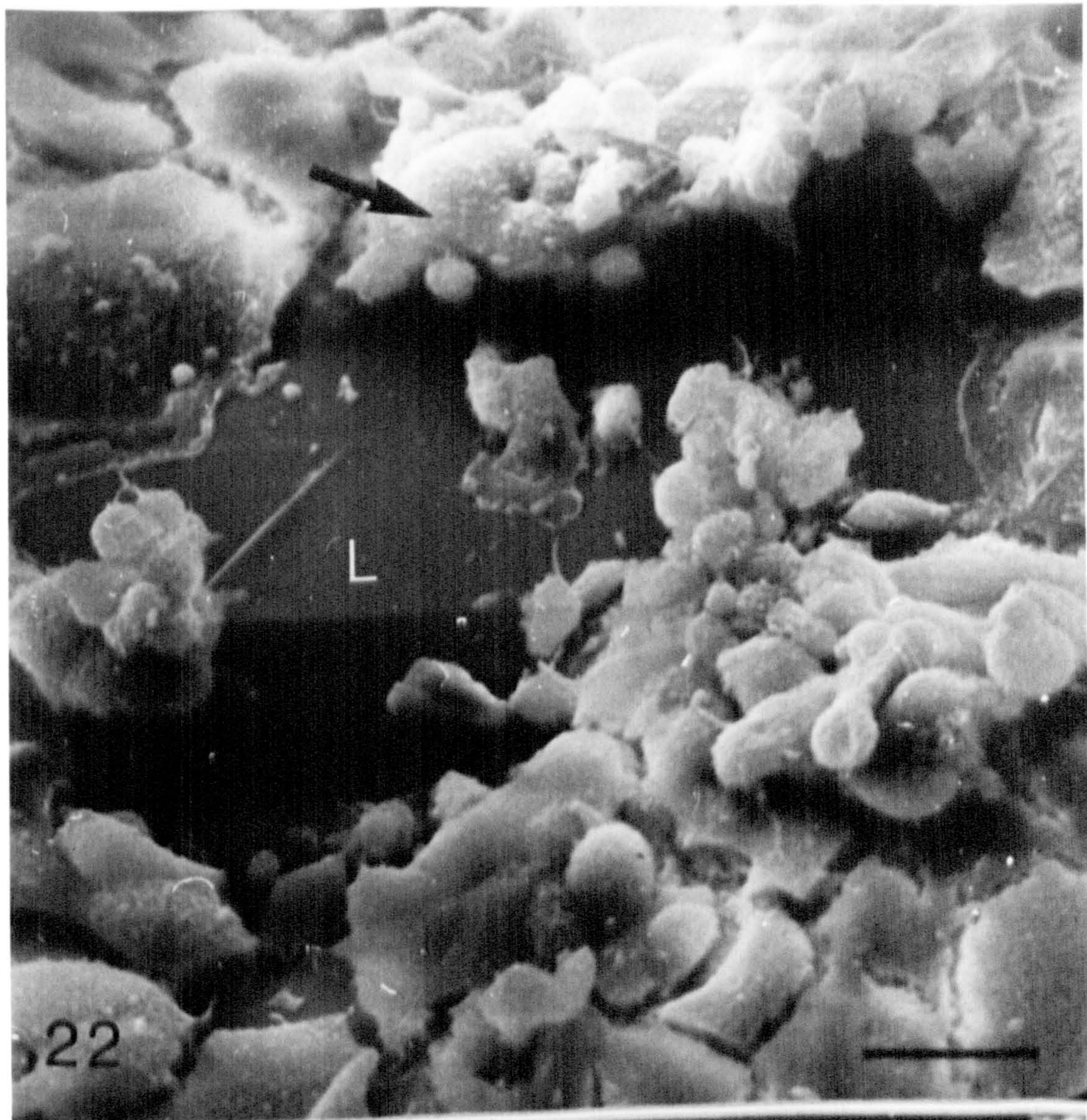




Fig. 24. An amoeboid T. vaginalis which has extended a pseudopodium beneath an epithelial cell. Note the large lamella (la) which is part of the peripheral cytoplasm of a cell out of view in this figure. This figure should be examined in conjunction with Figs. 25 and 26.

Bar = 10  $\mu$ m.

Fig. 25. Same area as Fig. 24 but the specimen has been rotated through 150° in the SEM to show the pseudopodium of an amoeboid T. vaginalis lying on the substratum beneath an epithelial cell. (The cell has fractured during the critical point drying process).

Bar = 10  $\mu$ m.



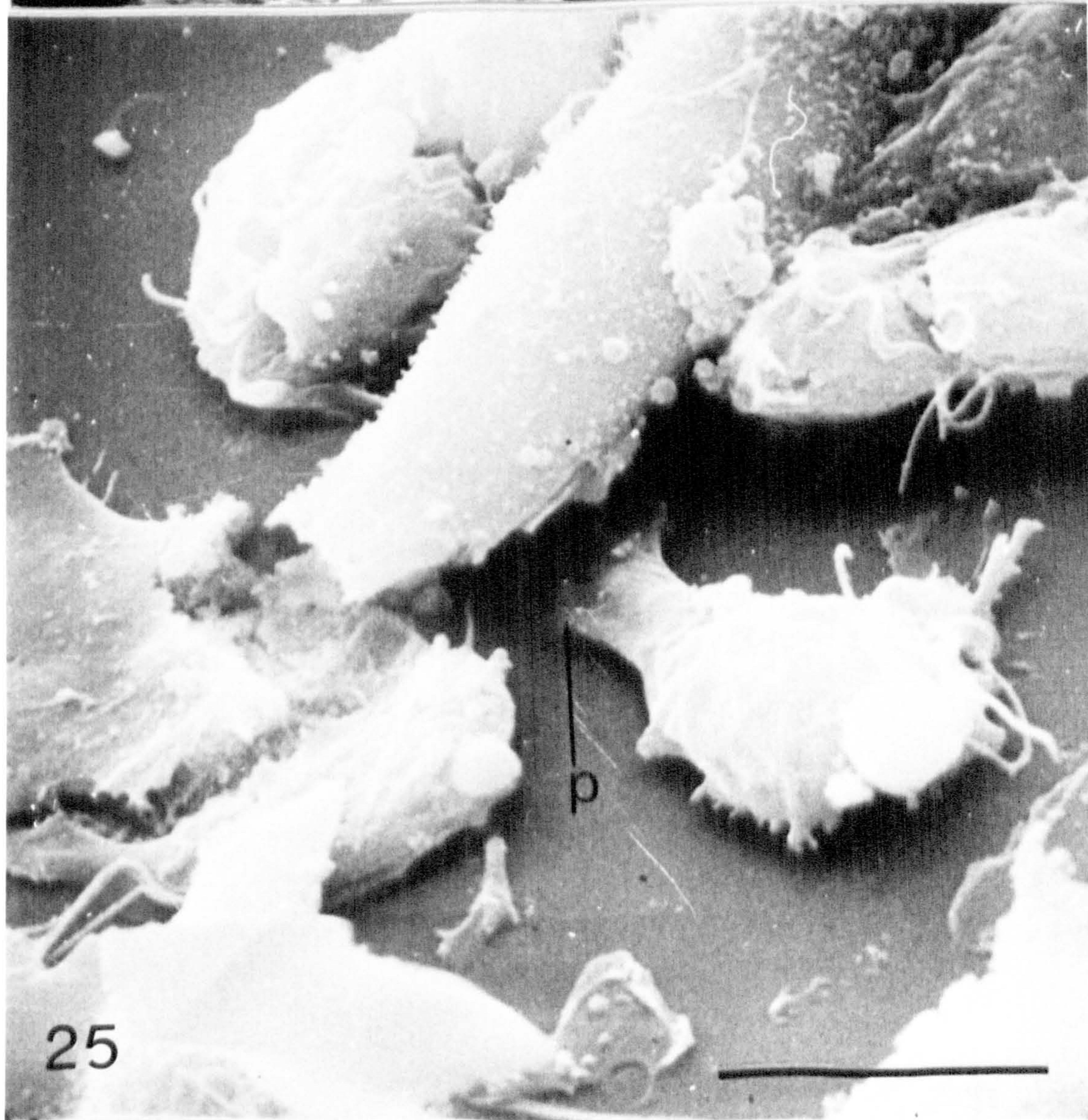
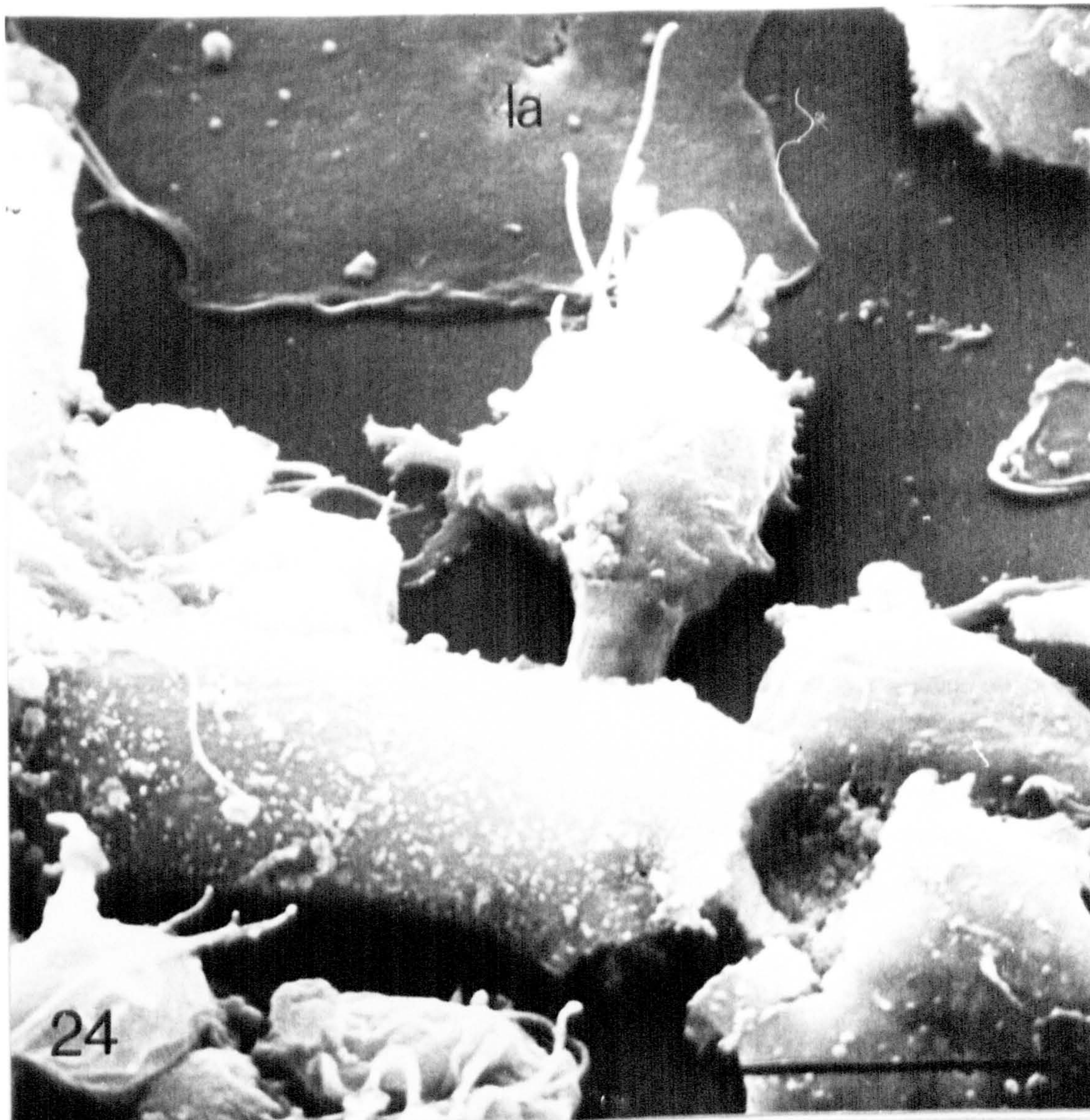




Fig. 26. Detail of Fig. 24 showing an amoeboid T. vaginalis which has extended a pseudopodium under an epithelial cell. Note the small pseudopodia at the rear of the parasite (small arrows). The surface of the large pseudopodium is smooth. The opening of a pinocytotic pit can be seen (large arrow). The vesicular material on the upper surface of the parasite is debris from lysed cells which has adhered to the parasite.

Bar = 3  $\mu$ m.

Fig. 27. Three T. vaginalis adhering to the sides of a rounded epithelial cell; the cell surface is covered with many microvilli.

Bar = 10  $\mu$ m.



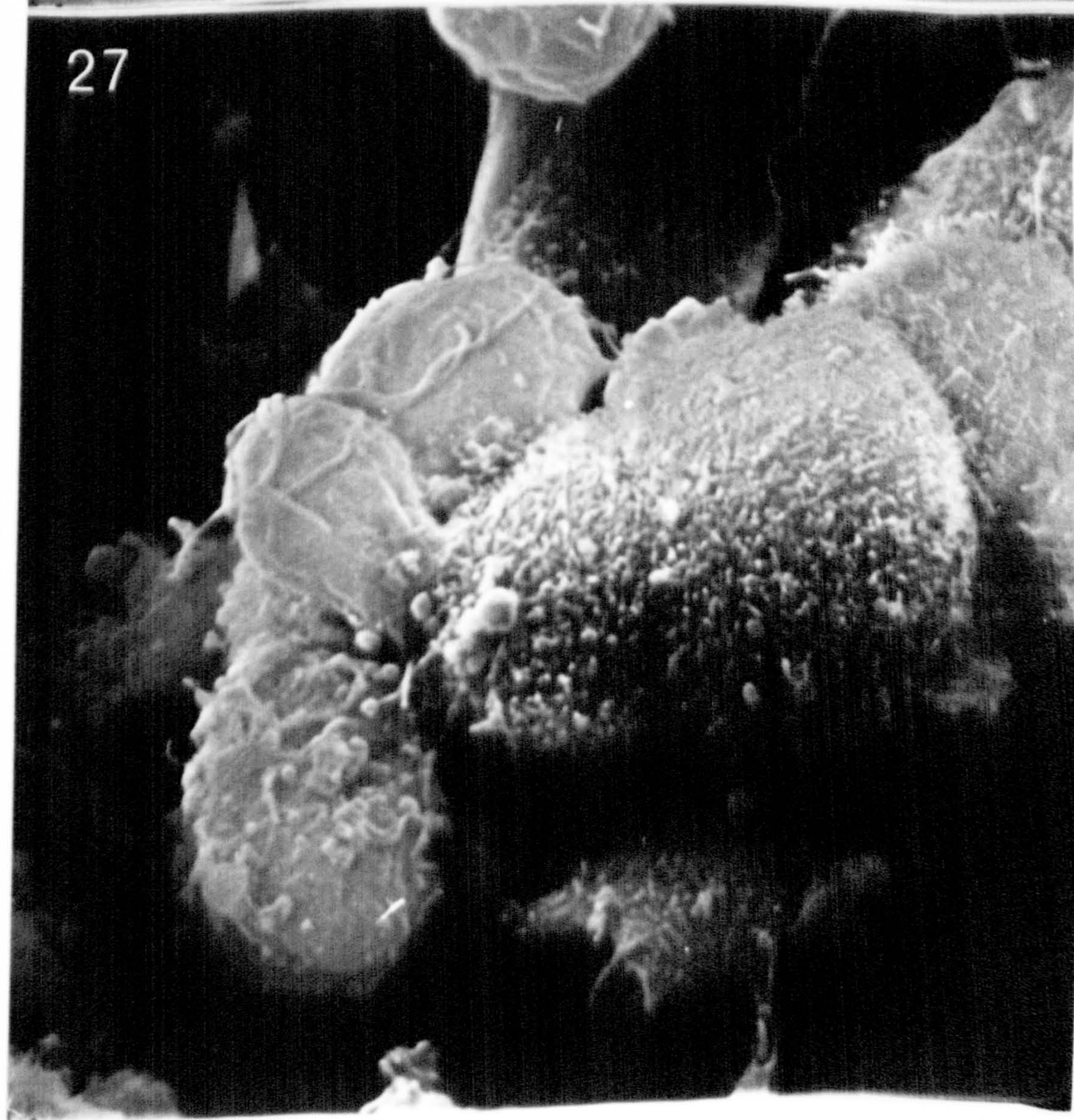
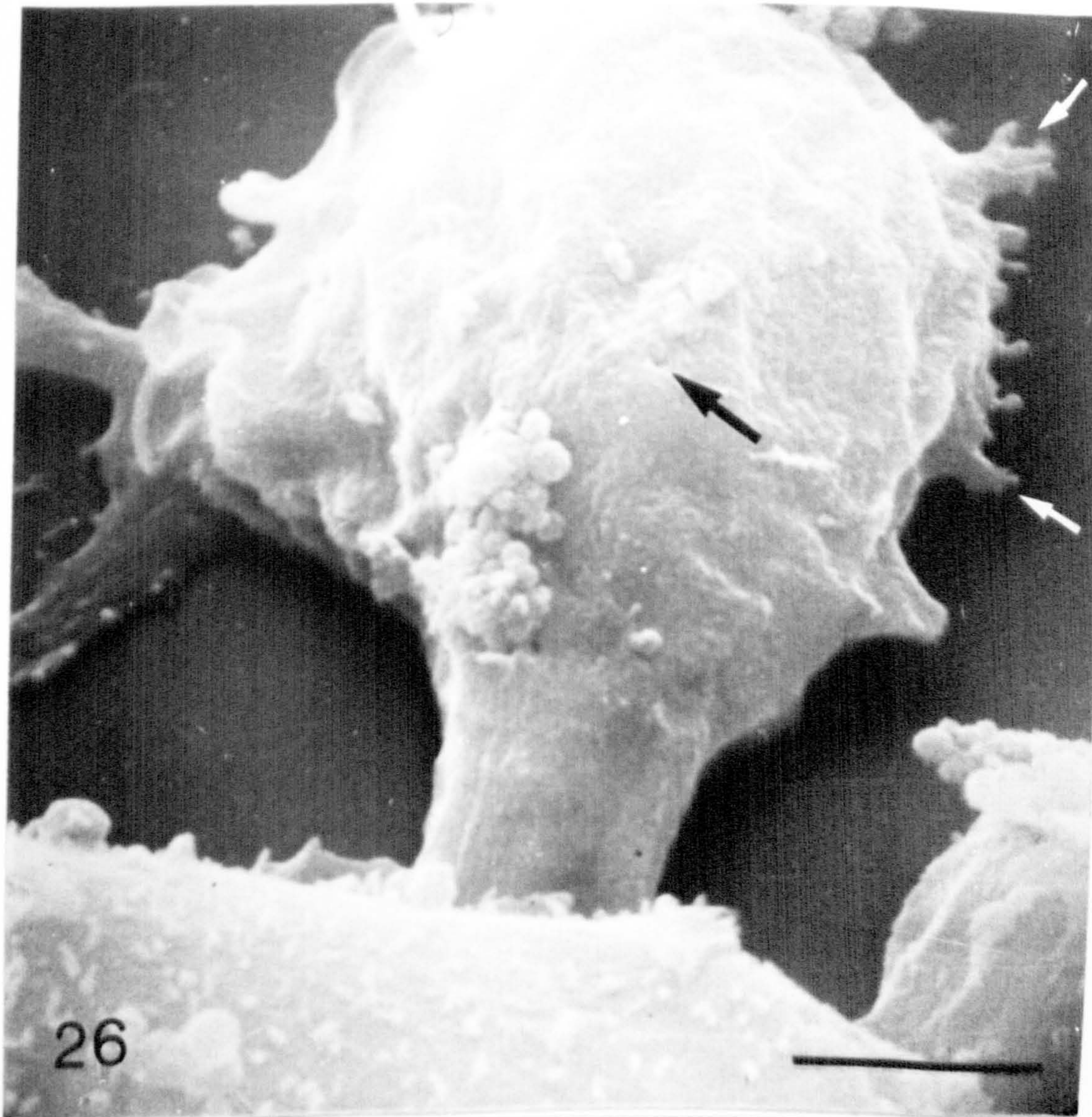




Fig. 28. A polarised amoeboid T. vaginalis adhering to the upper surface of an epithelial cell at the edge of a lesion. The organism is polarised along its longitudinal axis; two small pseudopodia (arrows) project forwards in front of the anterior flagella and are in close contact with the epithelial cell.

Bar = 5  $\mu$ m.

Fig. 29. T. vaginalis adhering to the side of an epithelial cell. Note that the flagella project up into the medium. The cell surface is covered with debris from lysed epithelial cells.

Bar = 5  $\mu$ m.



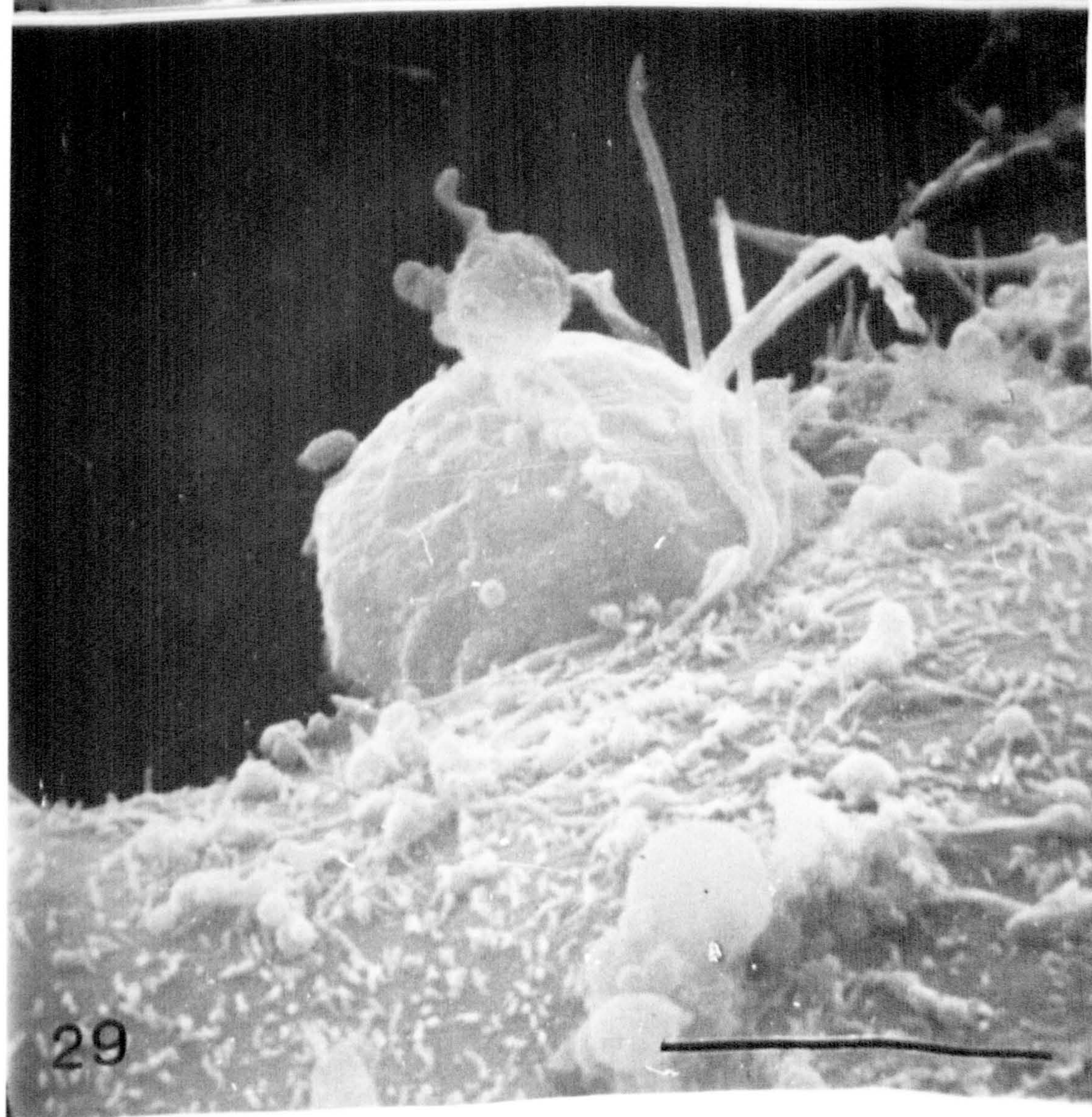
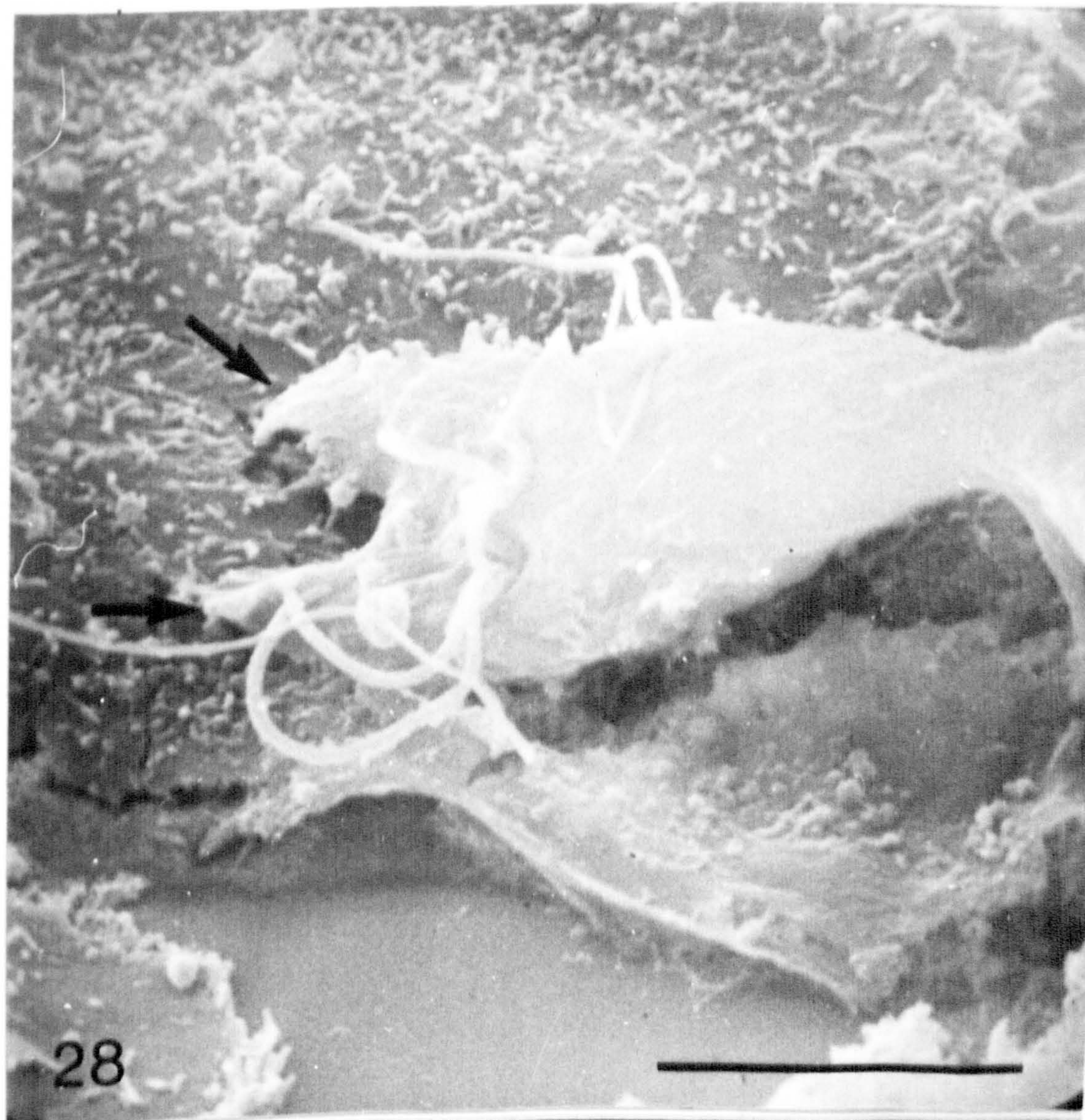




Fig. 30. Two trichomonads adhering to a group of epithelial cells. Note that one parasite (arrow) lies underneath the cells. The flagella and undulating membrane of the other parasite project out into the medium. S = substratum.

Bar = 5  $\mu$ m.

Fig. 31. T. vaginalis adhering to the side of an epithelial cell. The trichomonad has looped its flagella around a long retraction fibre (rt) which extends away from the cell over a cell-free area of the substratum. Bar = 5  $\mu$ m.



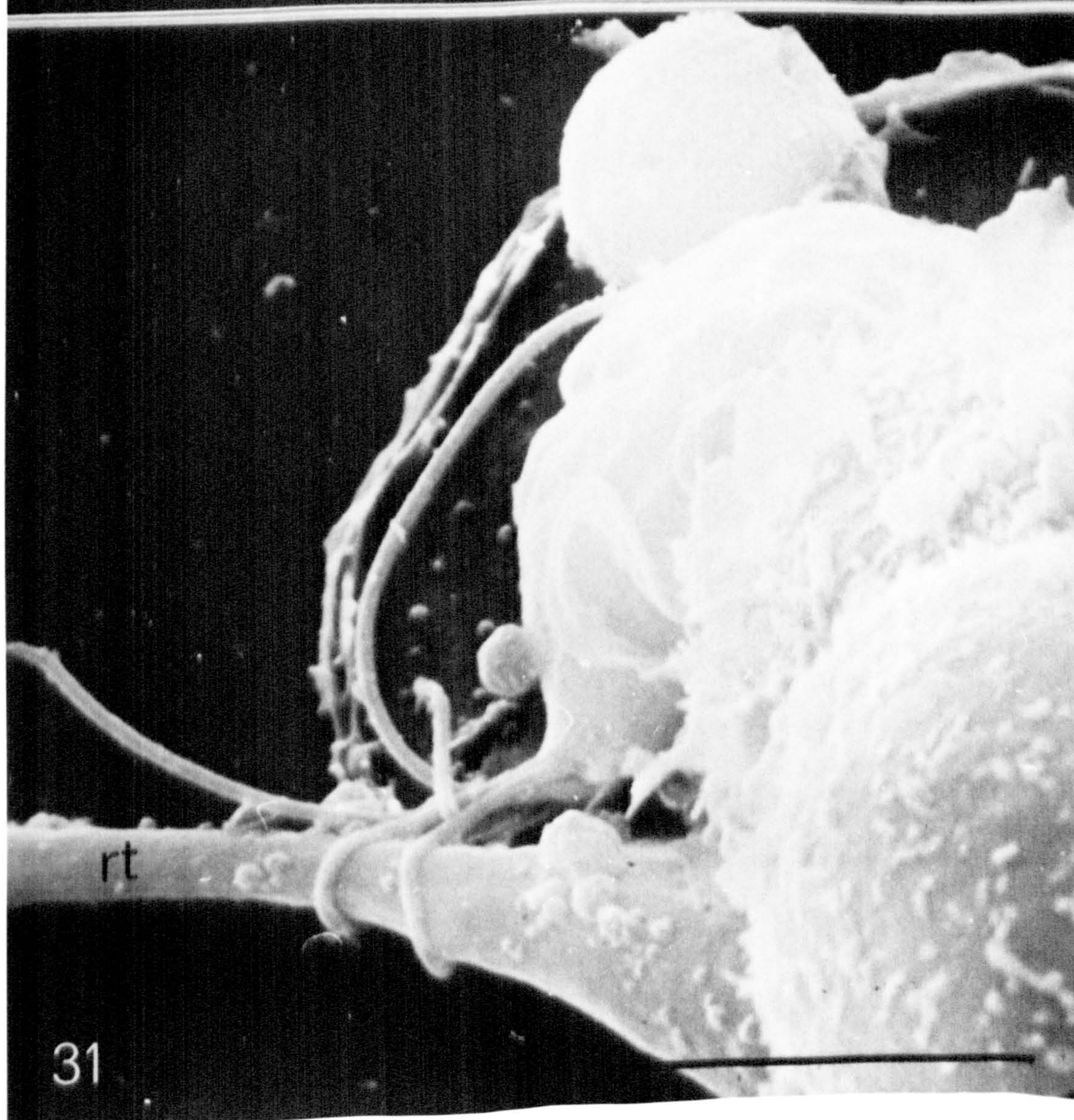
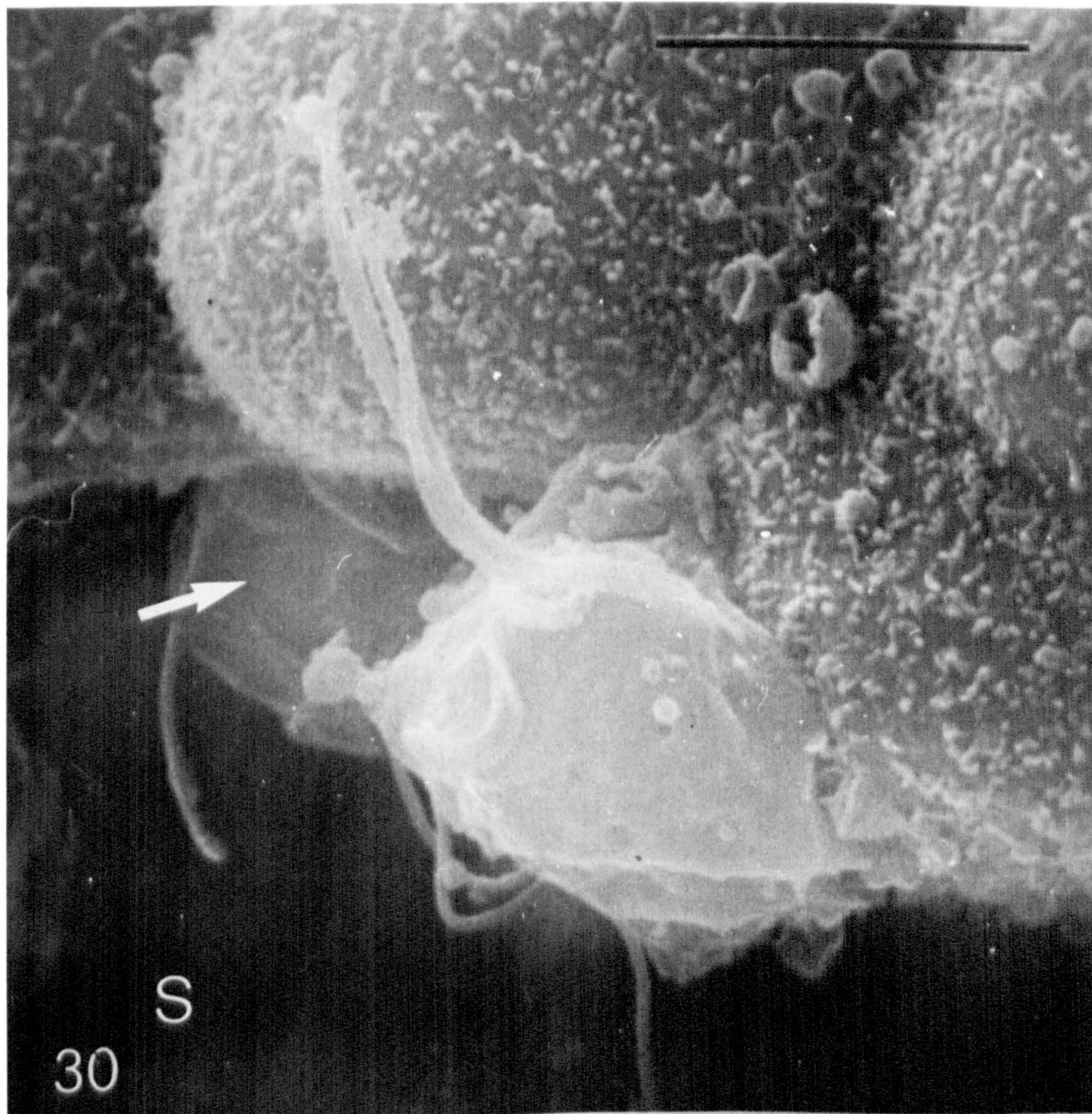




Fig. 32. An amoeboid T. vaginalis which appears to have been crawling over the surface of an epithelial cell at the time of fixation. The axostyle projects from the rear of the organism. Two broad flat pseudopodia extend over the cell. Note that this trichomonad possesses one anterior flagellum which does not arise, as normal, from the flagellar pocket (arrow).

Bar = 5  $\mu\text{m}$ .

Fig. 33. High magnification view of the region of contact between a pseudopodium of T. vaginalis and an epithelial cell. Note the microvilli on the cell's surface which are approximately 0.1  $\mu\text{m}$  in diameter and 0.6  $\mu\text{m}$  in length.

Bar = 2  $\mu\text{m}$ .



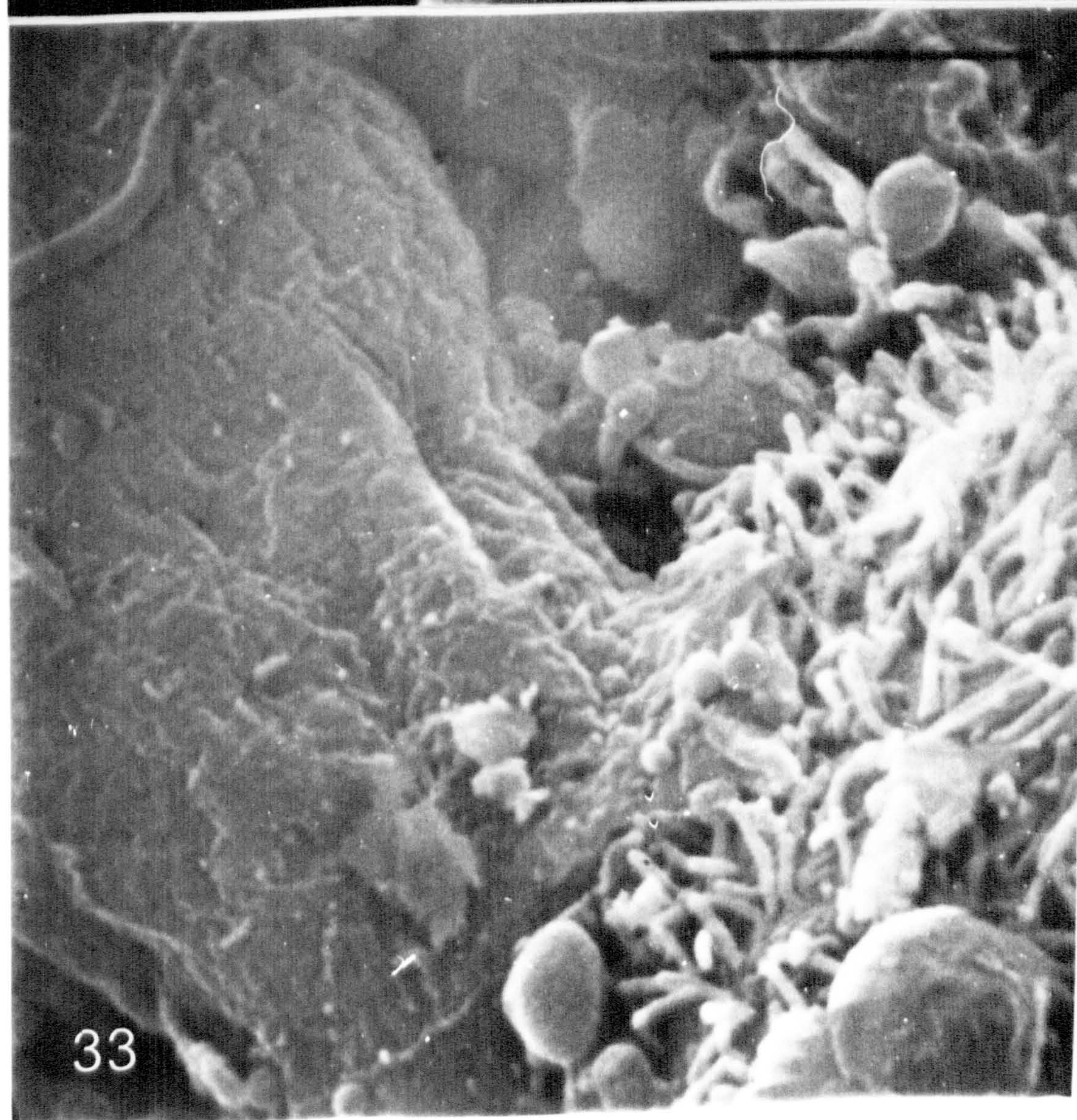
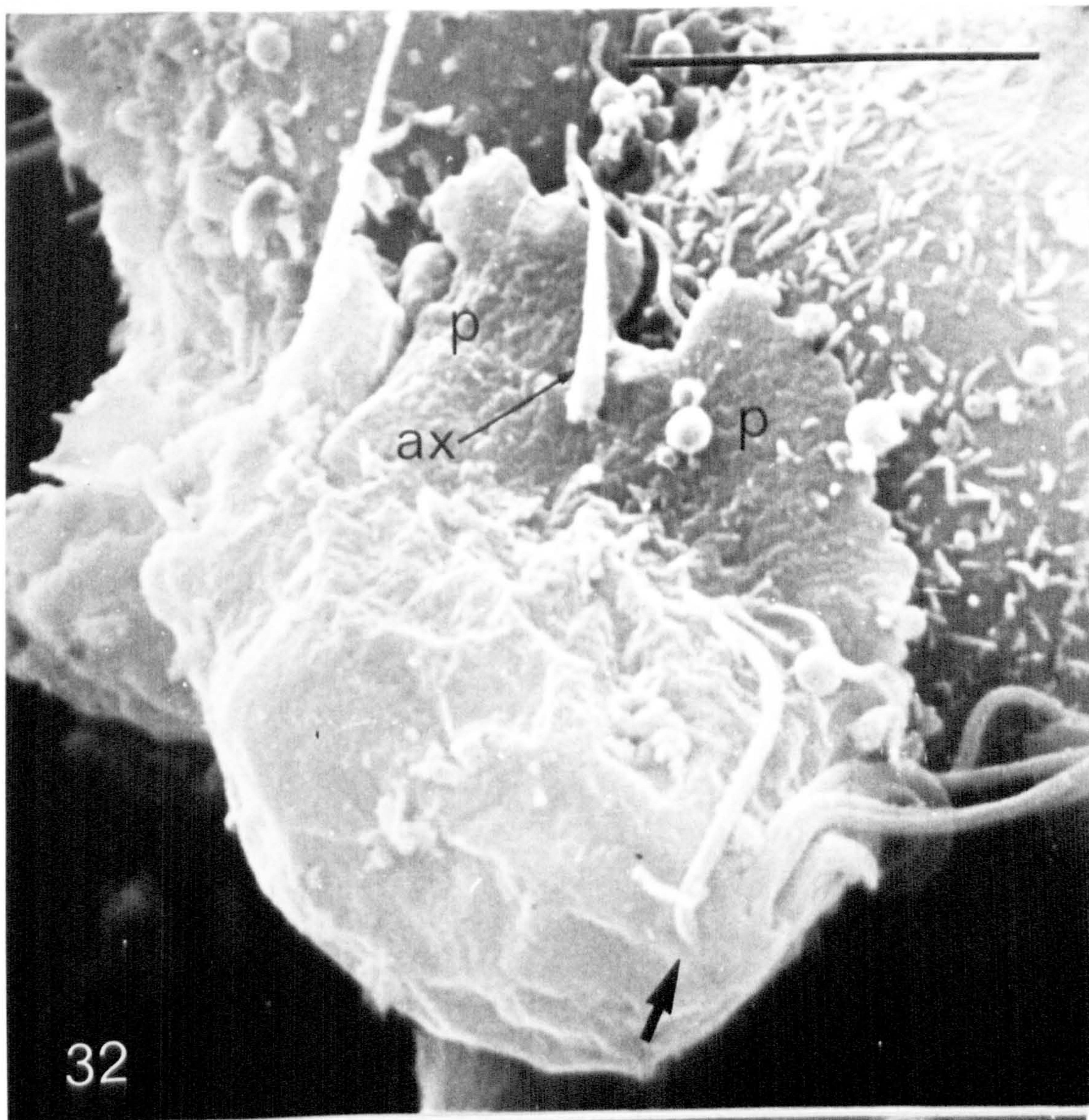




Fig. 34. T. vaginalis lying beneath the remains of a lysed epithelial cell. The small spherical bodies (arrows) 0.5 to 1.0  $\mu\text{m}$  in diameter may be mitochondria and/or vesicles, and the large body on the left, the nucleus. The nature of the smooth-surfaced body (b) is unknown but similar bodies were seen in cell cultures uninfected with parasites. Note that the cell on the lower right (ec) has lost most of its microvilli as compared with the upper cell.

Bar = 5  $\mu\text{m}$ .



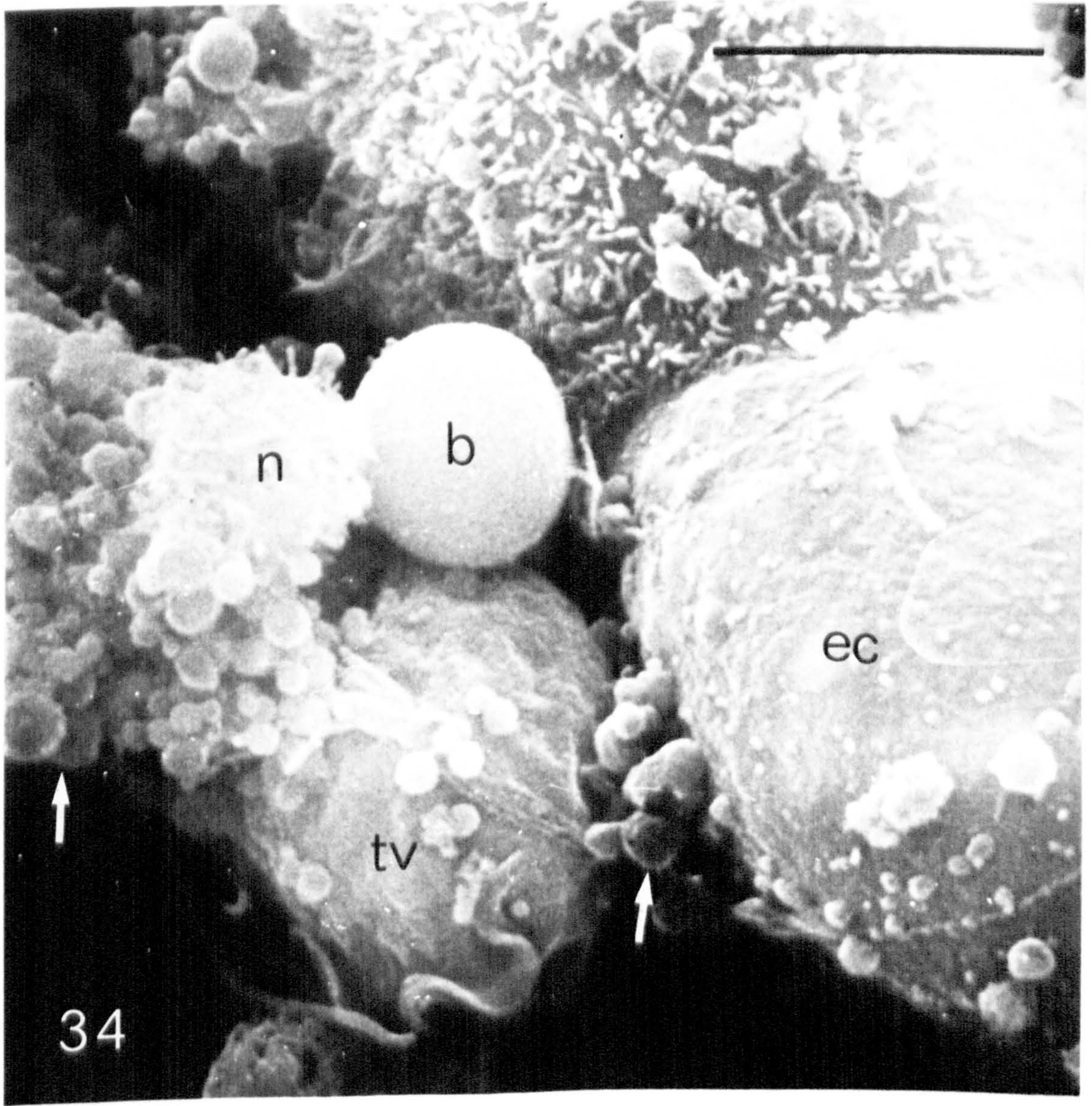




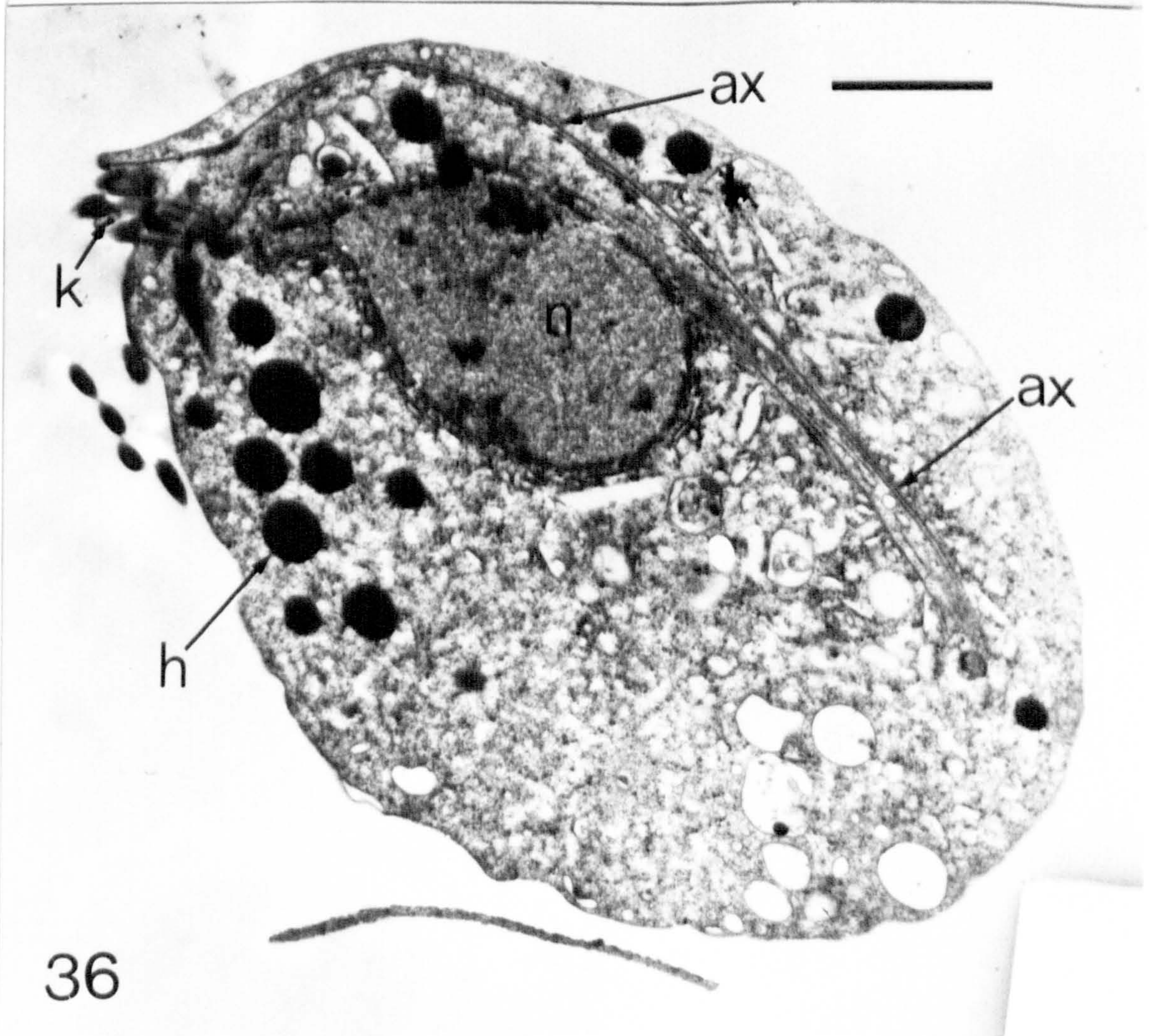
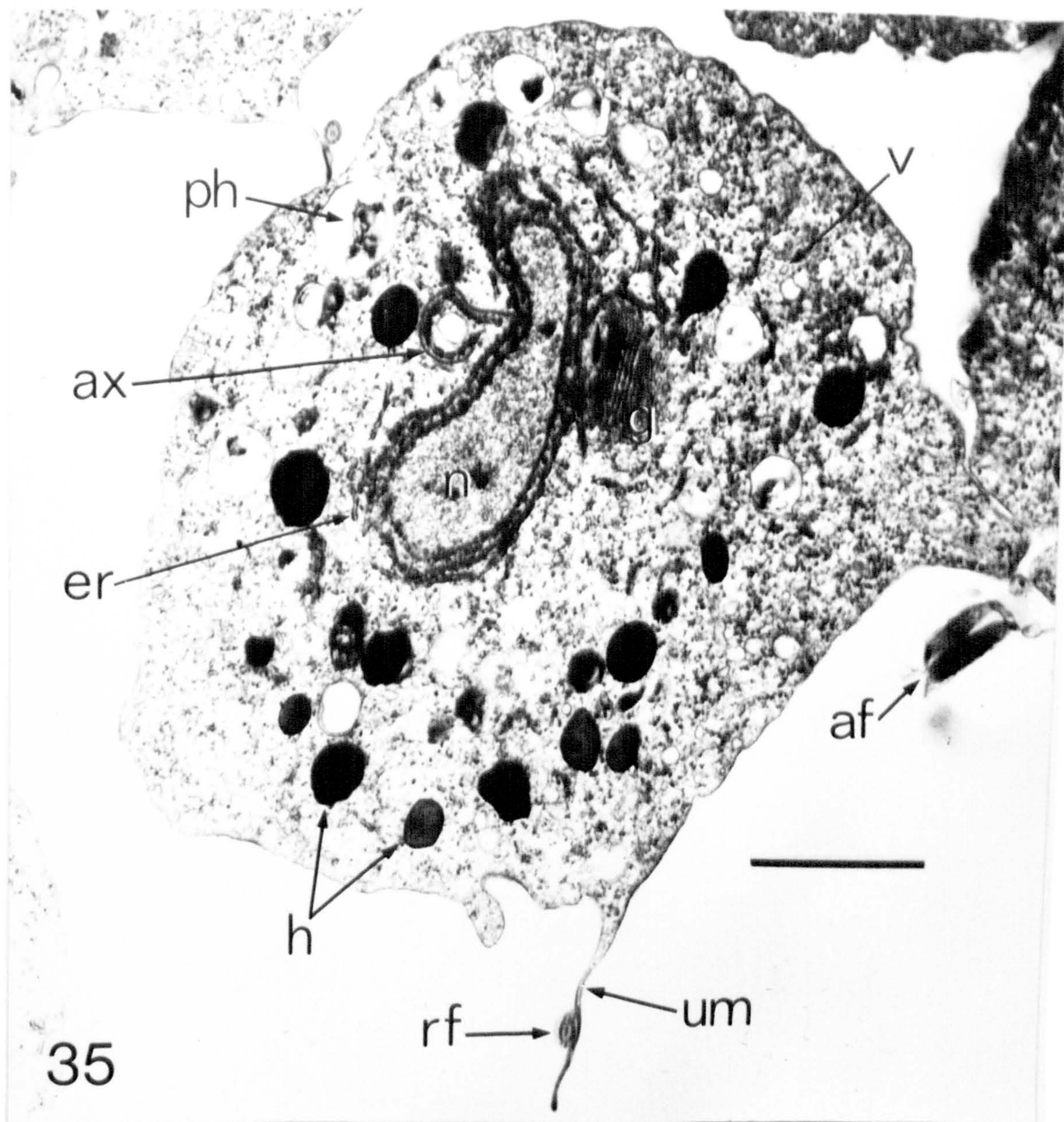
Fig. 35. Transmission electron micrograph of a transverse section through the mid-region of T. vaginalis. The nucleus (n) is surrounded by a corona of rough endoplasmic reticulum (er). The axostyle (ax) and Golgi body (g) lie on opposite sides of the nucleus. Parts of the anterior flagella (af) and the undulating membrane (um) are shown. The cytoplasm contains a population of electron-dense granules 0.5 to 1.0  $\mu\text{m}$  in diameter which are the hydrogenosomes (h), and a heterogeneous population of phagosomes (ph) and vesicles (v).

Bar = 2  $\mu\text{m}$ .

Fig. 36. Longitudinal section through T. vaginalis showing the kinetosomes (k) of the anterior flagella, the nucleus (n) and the axostyle (ax) which passes posteriorly from the kinetosomes to the posterior pole of the body. h = hydrogenosome.

Bar = 2  $\mu\text{m}$ .







Figs. 37 to 39. Electron micrographs of thin sections through T. vaginalis showing the fine structure of some of the organelles.

Fig. 37. Transverse section through the undulating membrane and re-current flagellum. The flagellum has the normal 9 + 2 arrangement of microtubules (t) and is attached to the undulating membrane at a point about 500 nm from its free edge. Note the electron-dense marginal lamella (ml) within the distal portion of the undulating membrane. There is a gap of 6 nm between the flagellum and undulating membrane which does not contain any electron-dense material. h = hydrogenosome; gl = glycogen granule; r = ribosomes.

Bar = 250 nm

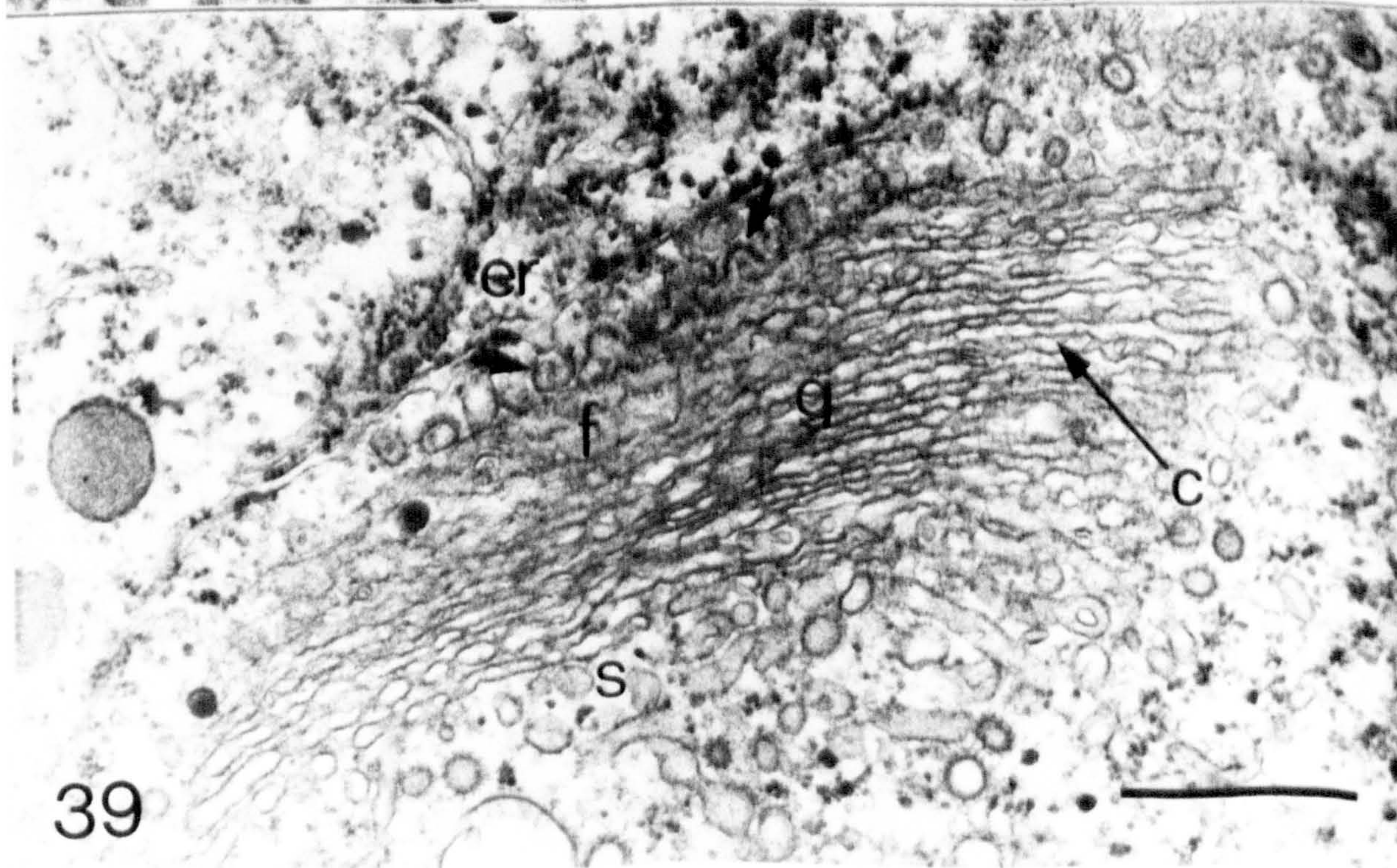
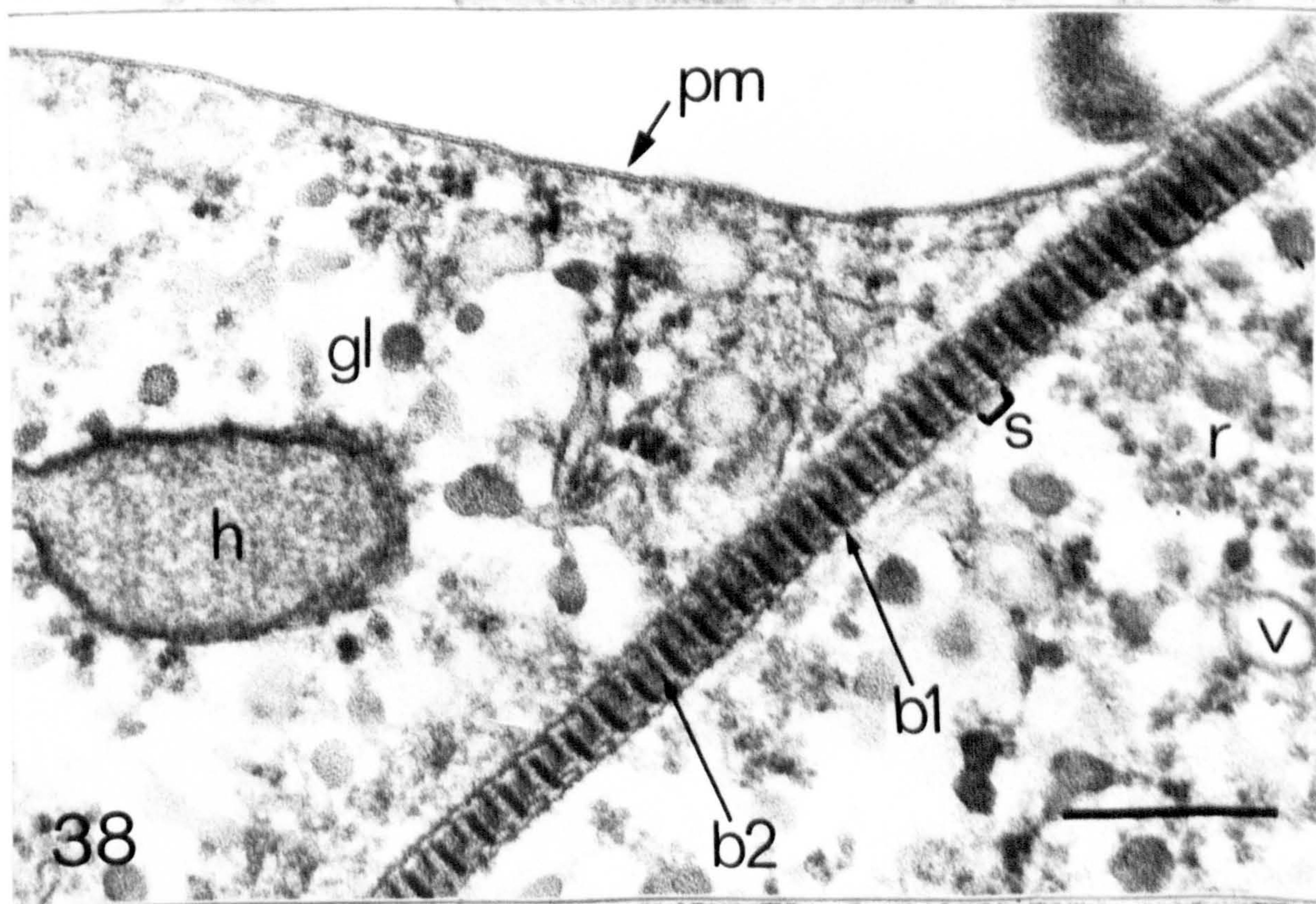
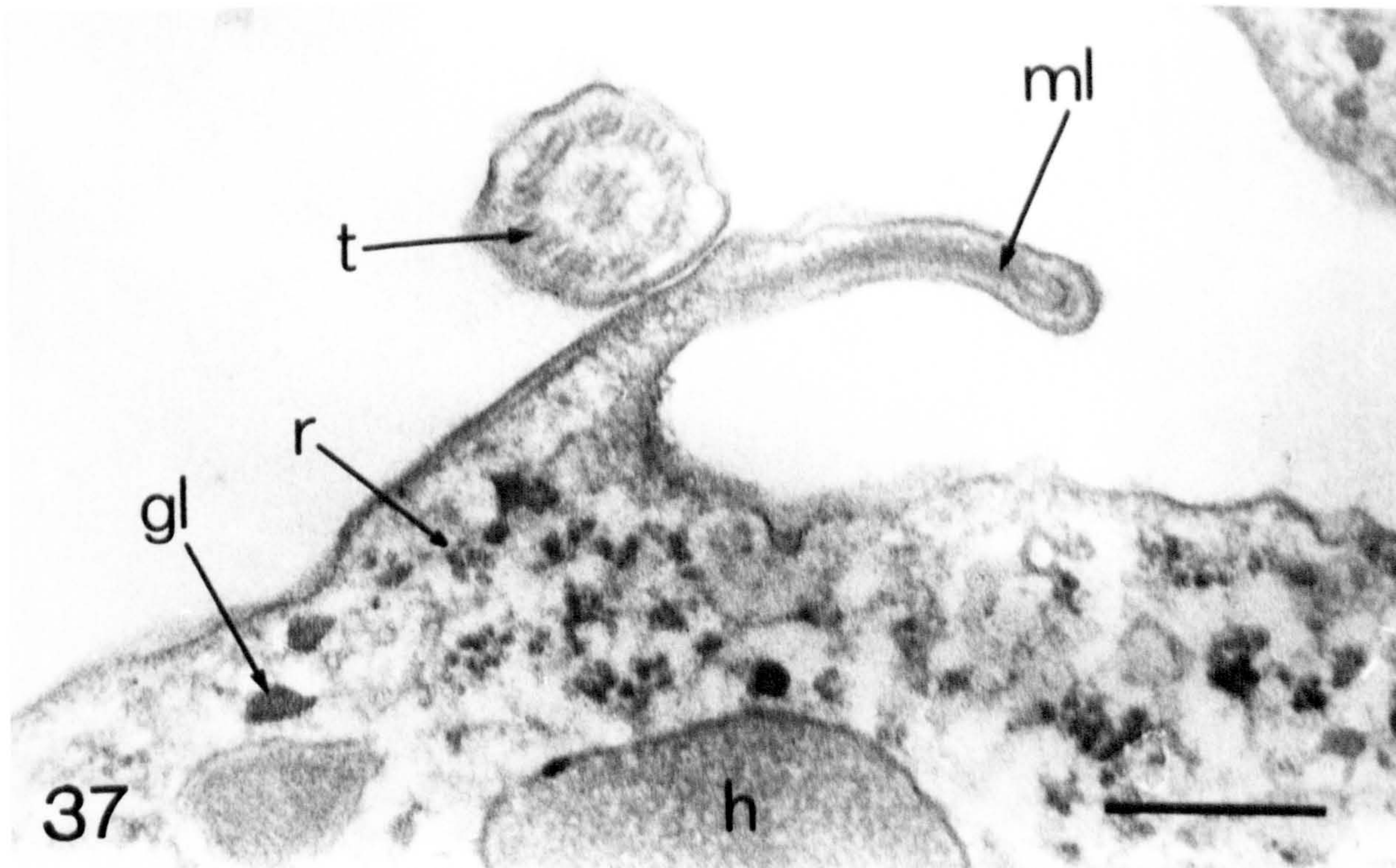
Fig. 38. Costa and plasma membrane. The costa is a filamentous rod which lies beneath the undulating membrane. The costa is divided transversely into segments (s) by electron-dense bands (b1) 12 nm wide with a centre-to-centre spacing to 60 nm. Each segment contains longitudinally aligned filaments which are traversed by secondary electron-dense bands (b2). The plasma membrane (pm) has a trilaminar structure and is 7 nm thick. Note that no surface coat is evident on the outer surface of the membrane in this section. gl = glycogen granules; r = ribosomes; h = hydrogenosome; v = vesicles.

Bar = 250 nm

Fig. 39. Golgi body and endoplasmic reticulum. The Golgi body (g) consists of many parallel membrane-bound cisternae (c). The forming face (f) lies close to the endoplasmic reticulum (er) from which vesicles (arrows) appear to pass and fuse with the forming face of the Golgi. On the opposite face, the secretory face (s), the cisternae break up into vesicles 50 to 100 nm in diameter which are released into the cytoplasm.

Bar = 500 nm







Figs. 40 to 42. Interdigitation-type contacts between T. vaginalis in infected epithelial cell cultures.

Fig. 40. Two T. vaginalis showing the reciprocal interdigitation of pseudopodia (p) in the area of contact.

Bar = 3  $\mu$ m

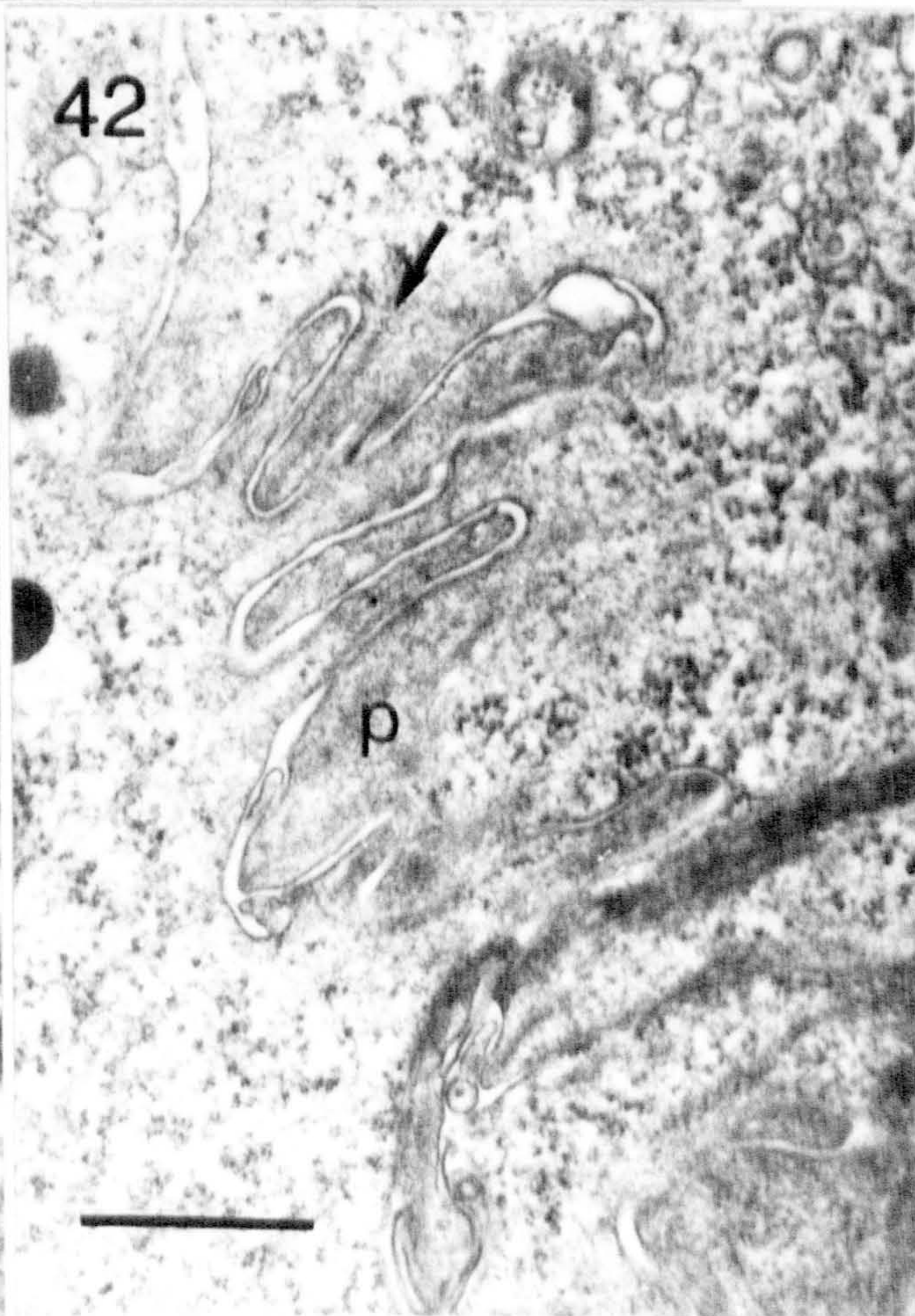
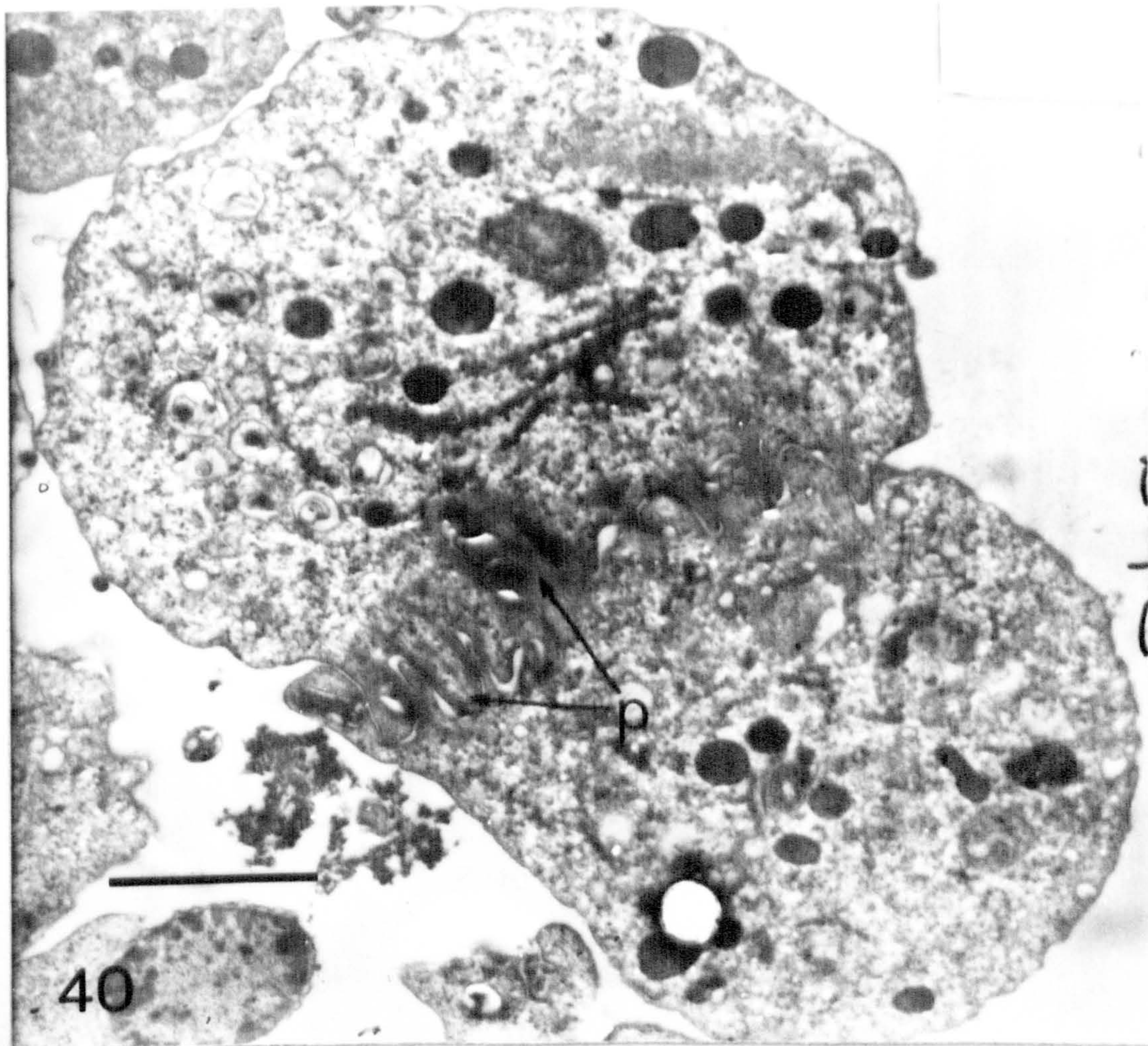
Fig. 41. Detail of Fig. 40, showing bundles of microfilaments within the pseudopodia of T. vaginalis. The bundles are oriented along the long axis of the pseudopodium (arrows) and perpendicularly to the surface of the adjacent organism.

Bar = 1  $\mu$ m

Fig. 42. Reciprocal interdigitation of pseudopodia of two T. vaginalis. A bundle of microfilaments lies in the centre of one pseudopodium (arrow). The apposed plasma membranes of the two trichomonads are separated by a gap of 20 to 50 nm in the region of interdigitation. Note the exclusion of vesicles and ribosomes from the pseudopodia, which contain a filamentous meshwork.

Bar = 1  $\mu$ m







Figs. 43 and 44. Specialised contacts between T. vaginalis

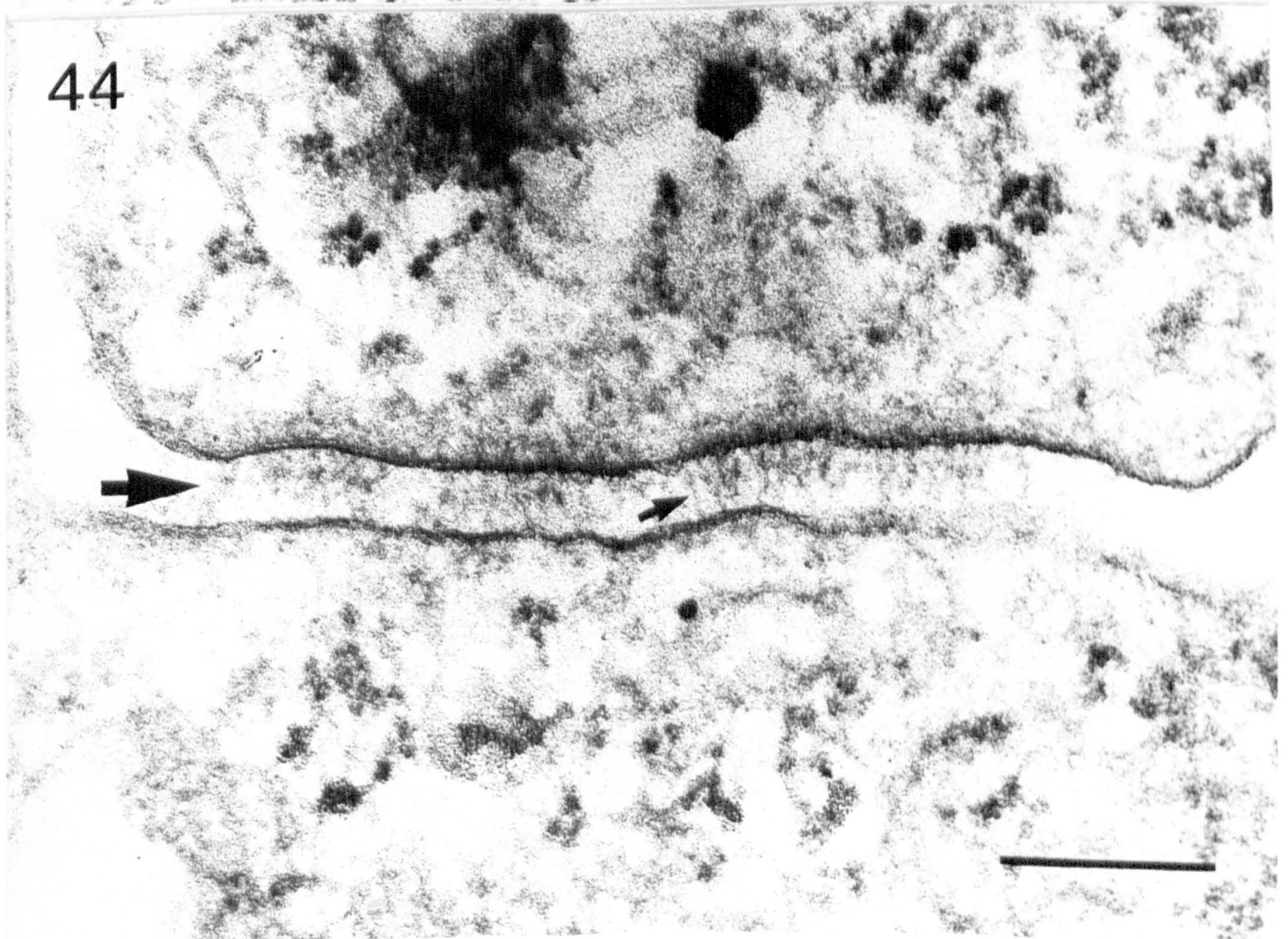
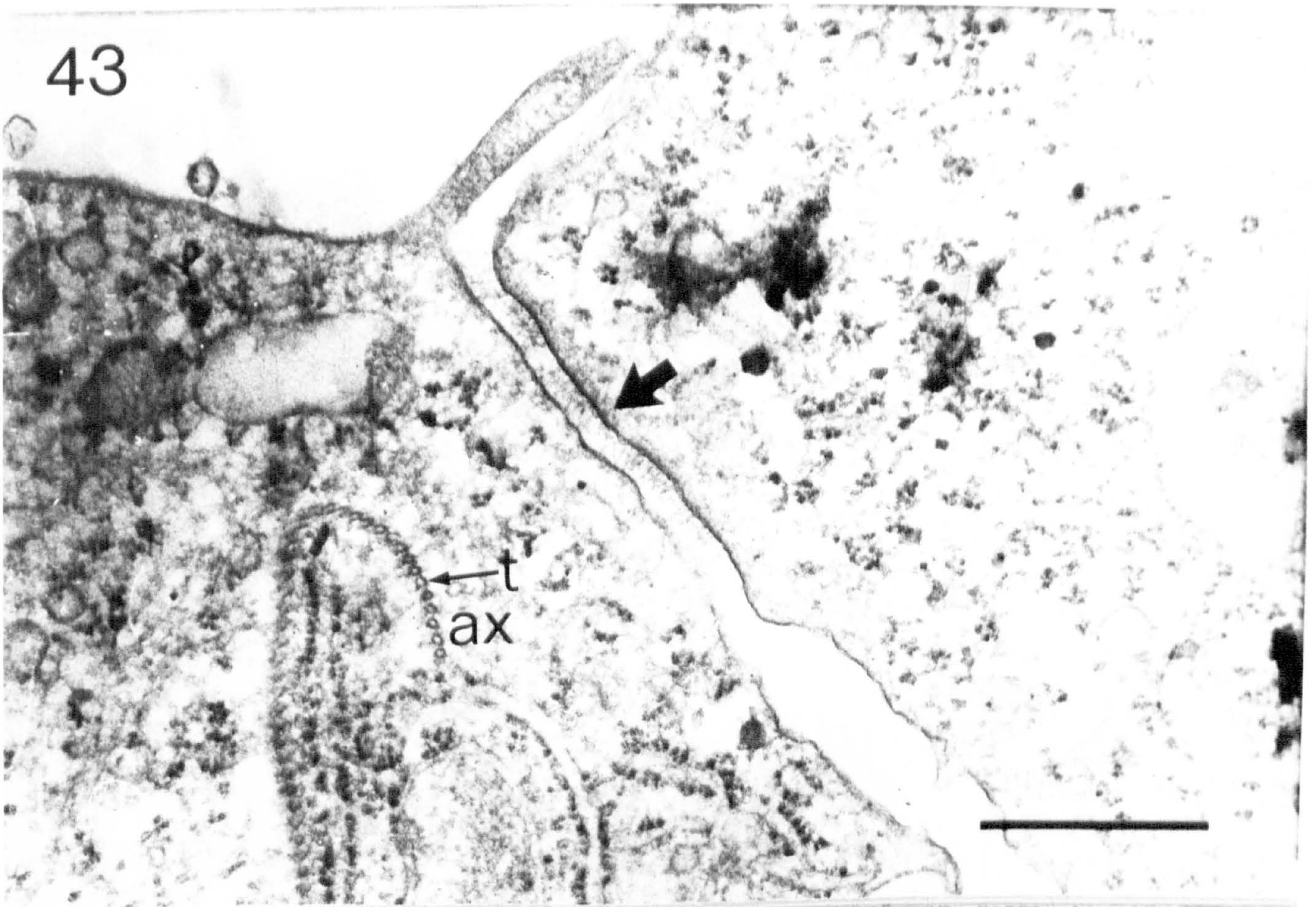
Fig. 43. Section through two T. vaginalis showing a region of close contact (arrow) which is characterised by a parallel apposition of plasma membranes over a distance of 1  $\mu\text{m}$ ; the membranes are separated by a gap of 75 nm which contains filamentous material. The section has passed through the axostyle (ax) of one trichomonad and shows the sheet of 22 nm diameter microtubules (t) in transverse section.

Bar = 500 nm

Fig. 44. Detail of Fig. 43 showing the specialised contact at high magnification. Lying in the gap between the apposed membranes is a central electron dense band (large arrow) which is traversed by filaments (small arrow) 5 to 10 nm in diameter which stretch between the membranes.

Bar = 200 nm.







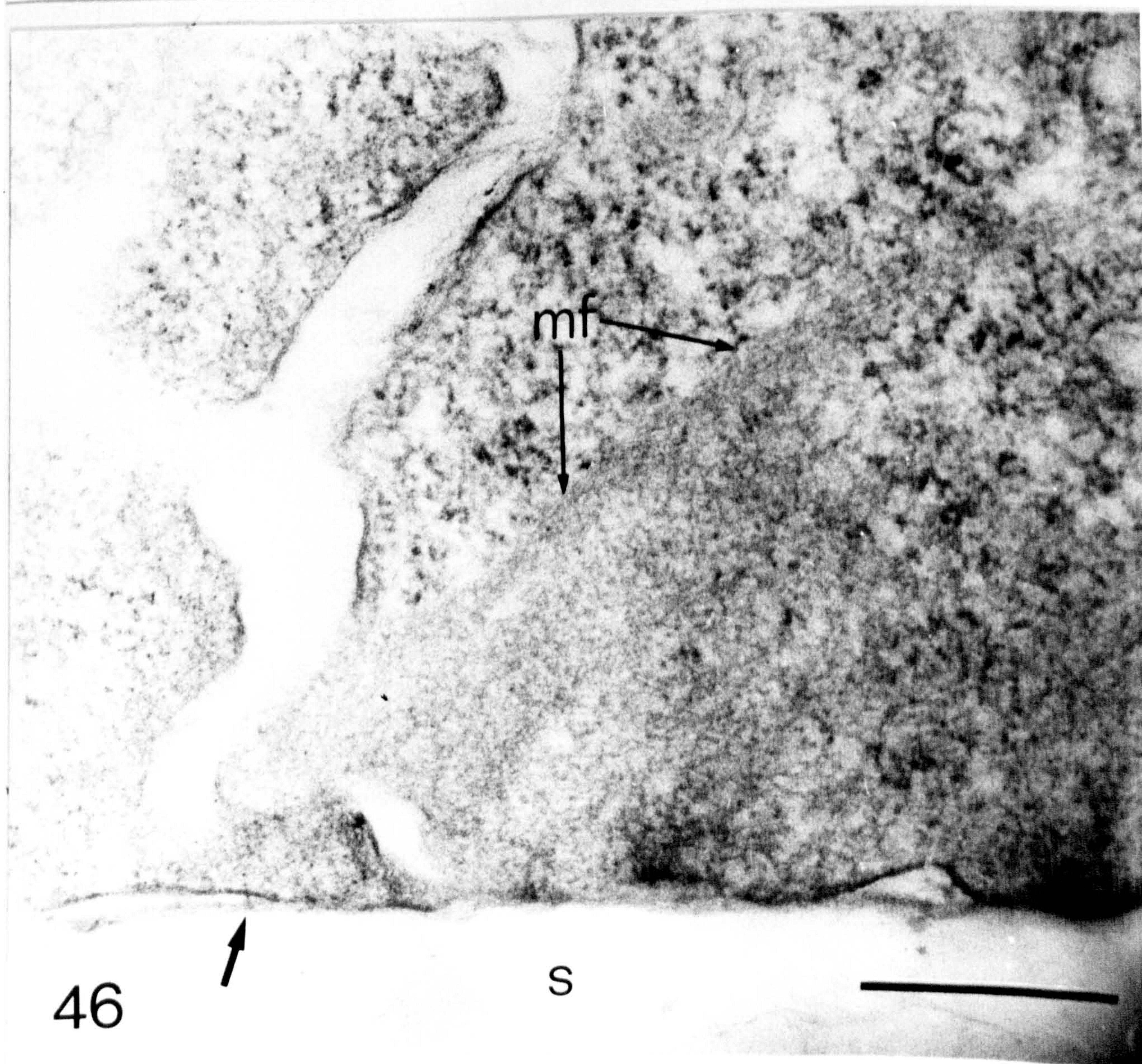
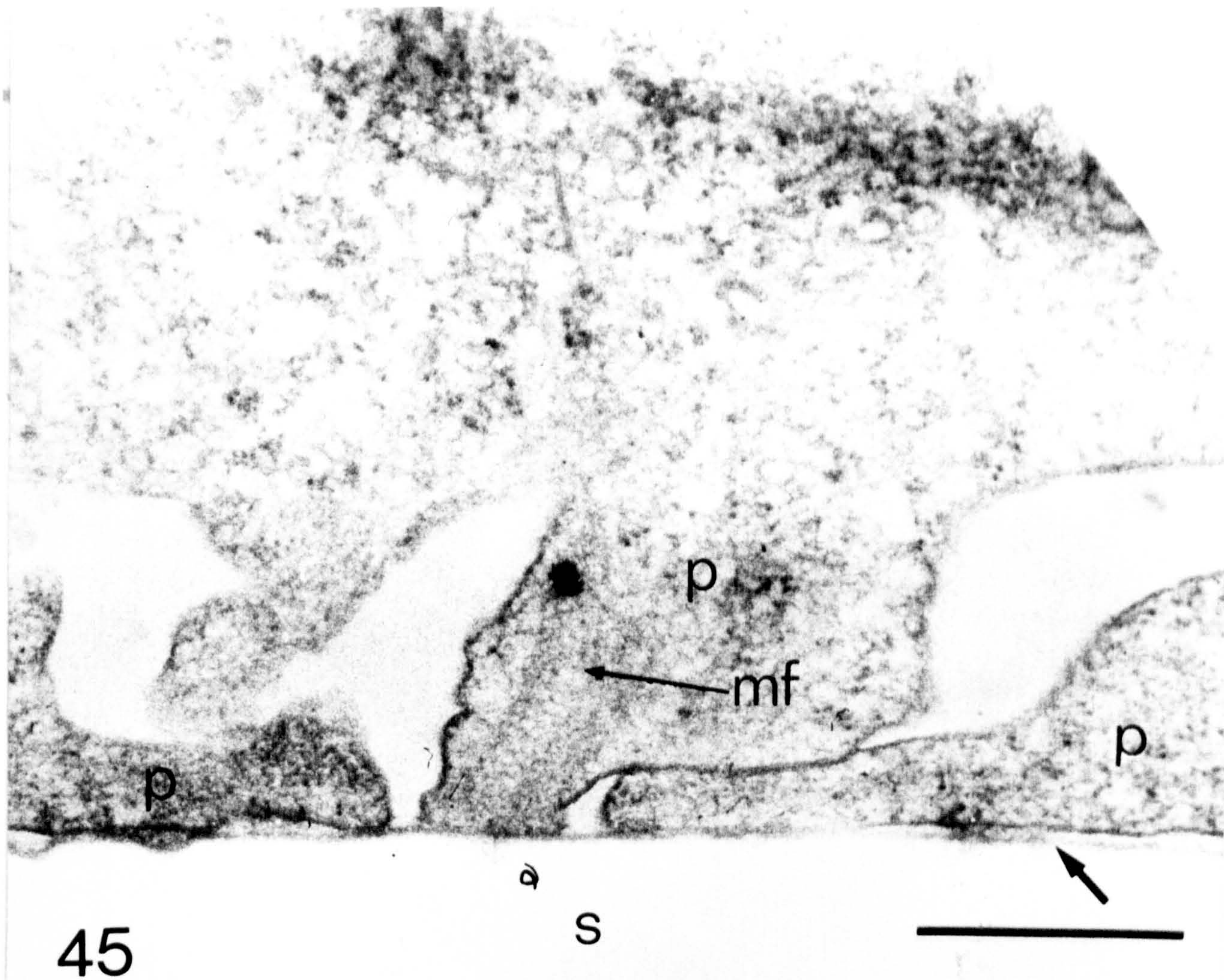
Figs. 45 and 46. Contacts between T. vaginalis and the substratum

Bars = 500 nm

Fig. 45. Vertical section, perpendicular to the substratum, through the lower surface of T. vaginalis showing three pseudopodia (p) which are in close contact with the substratum (s). The former position of the glass substratum is marked by an electron-dense line (arrow) which is probably composed of serum proteins adsorbed from the culture medium. The pseudopodia contain a meshwork of filamentous material (mf) which, in the central pseudopodium, contains tracts or bundles oriented towards the substratum. The plasma membrane of the trichomonad is separated from the substratum by a gap of not more than 30 nm (arrow).

Fig. 46. Vertical section through a pseudopodium at its point of contact with the substratum. Note the oriented bundles of microfilaments (mf) which pass obliquely away from the substratum, and the 30 nm gap (arrow) between the trichomonad and the substratum







Figs. 47 to 49. Vertical sections through a 3 day old monolayer (ecm) of RK 13 epithelial cells, illustrating the cellular fine structure.

Fig. 47. The medium facing, or upper, surfaces of the cells are covered with microvilli (mv). The lower surfaces of the cells are closely adherent to the substratum (s).

n = nucleus; nl = nucleolus; va = vacuole; med = medium

Bar = 5  $\mu$ m

Fig. 48. In many areas of the monolayer (ecm) lamellar processes of the peripheral cytoplasm of cells out of the plane of the section are seen lying beneath other cells. The presence of these processes create small spaces (arrow) between the lower surfaces of the monolayer and the substratum.

Bar = 5  $\mu$ m

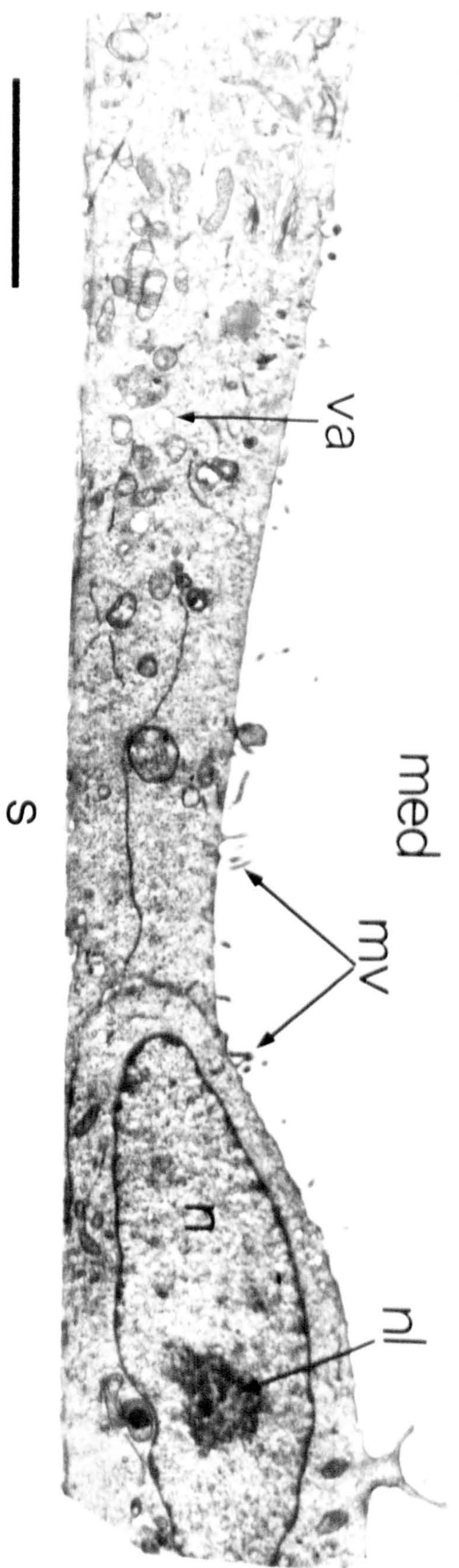
Fig. 49. Lateral adhesions of epithelial cells. Tight junctions (tj) are found on the medium side of the monolayer. Intermediate-type junctions (ij) are characteristically found closer to the substratum-apposed surfaces of the monolayer in areas of lateral interdigitation of the cells' surfaces. The adhesions of the cells to the substratum are usually associated with a mat of microfilaments (mf).

m = mitochondrion

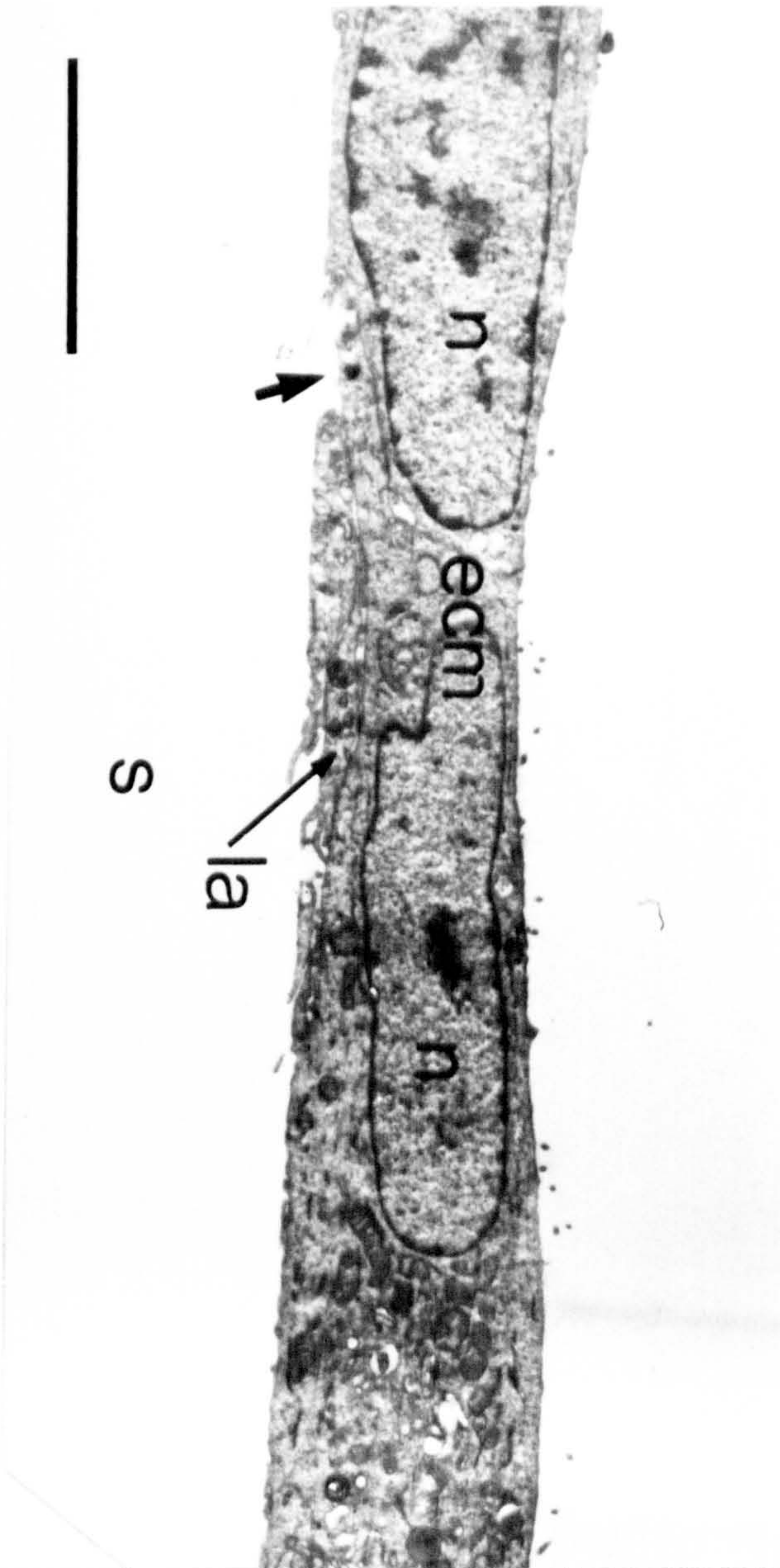
Bar = 2  $\mu$ m



47



48



49

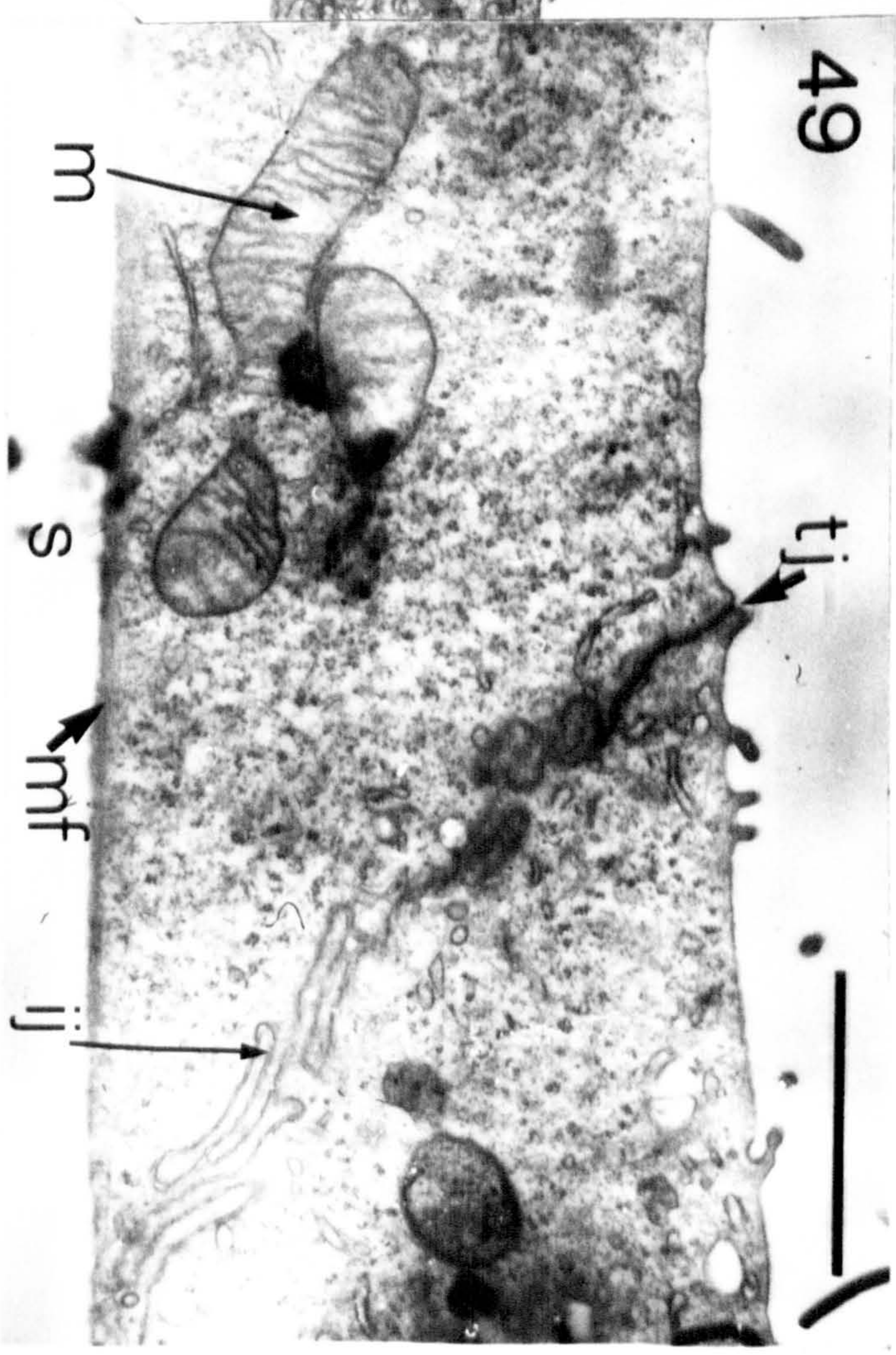
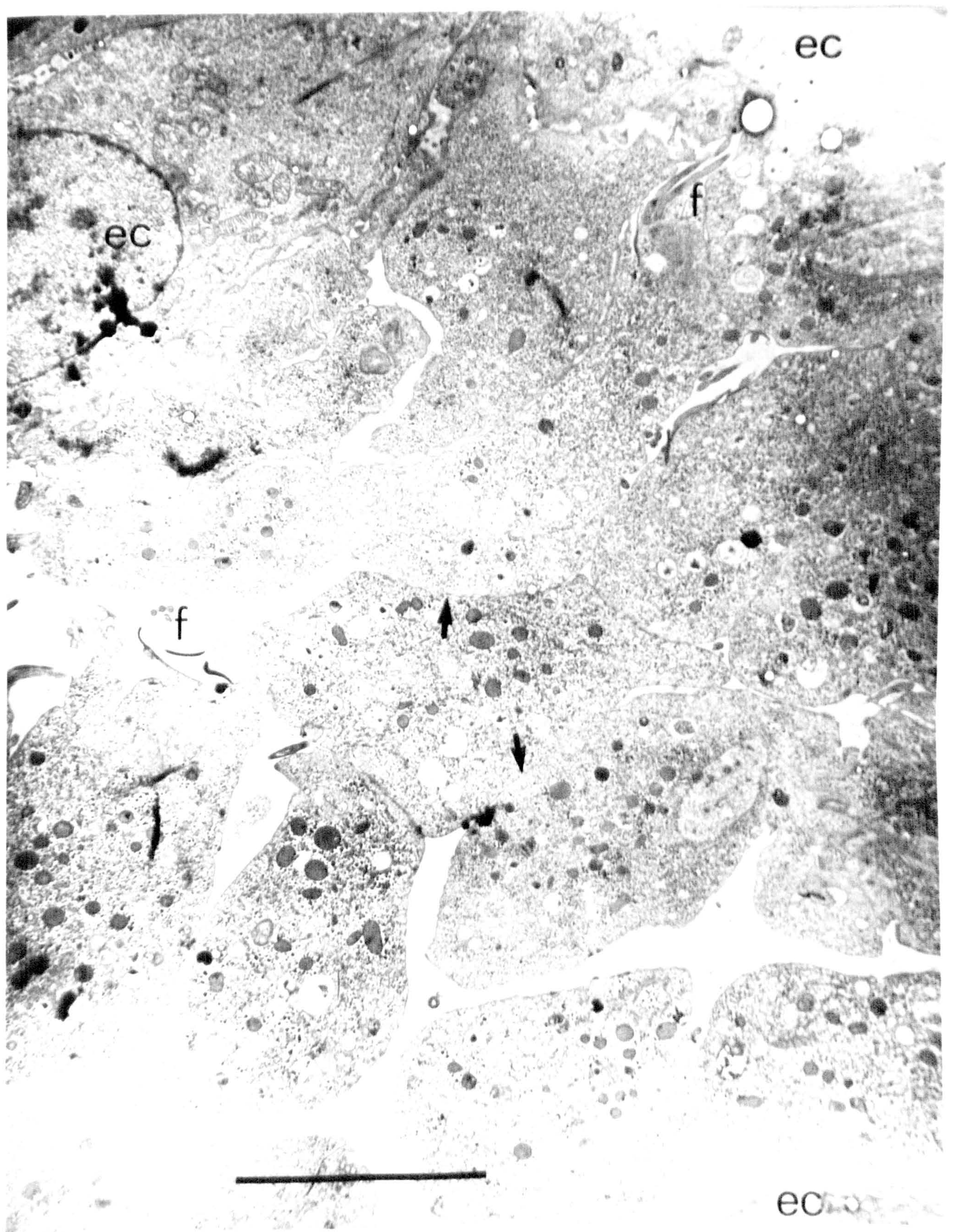




Fig. 50. Horizontal section, taken parallel to the substratum, through the edge of a lesion in a monolayer of epithelial cells infected for 6 h with T. vaginalis. Parts of cells (ec) can be seen at top left and right, and bottom right. The edge of the lesion is packed with T. vaginalis some of which are lying in close (about 50 nm) contact with their neighbours (arrows). Note the flagella (f) lying in the larger spaces between the trichomonads. At the top of the figure the trichomonads are in close contact with the epithelial cells.

Bar = 10  $\mu$ m







Figs. 51 to 53. Vertical sections, taken perpendicular to the substratum, through the edges of lesions in epithelial cell monolayers (ecm). Note that the electron dense line which generally marked the interface between the Araldite and glass substratum is absent in these sections.

Fig. 51. Two trichomonads lying between the substratum and the lower surfaces of two epithelial cells. The trichomonads appear to be compressed between the cells and the substratum; their lower surfaces are flattened against the substratum (small arrows) and their upper surfaces are in close contact with the cells (large arrow). Note that the epithelial cells appear undamaged.

Bar = 5  $\mu$ m

Fig. 52. T. vaginalis lying beneath epithelial cells at the edge of a lesion. The parasites have completely lifted one cell away from the substratum.

Bar = 5  $\mu$ m

Fig. 53. A group of four T. vaginalis palisaded against the cells at one side of a lesion. The parasites have displaced two cells from the substratum which are rounded with an increased number of microvilli on their surfaces (arrows) as compared with a third cell which still adheres to the substratum. Note the large autophagosome (ph) within the latter cell.

Bar = 5  $\mu$ m



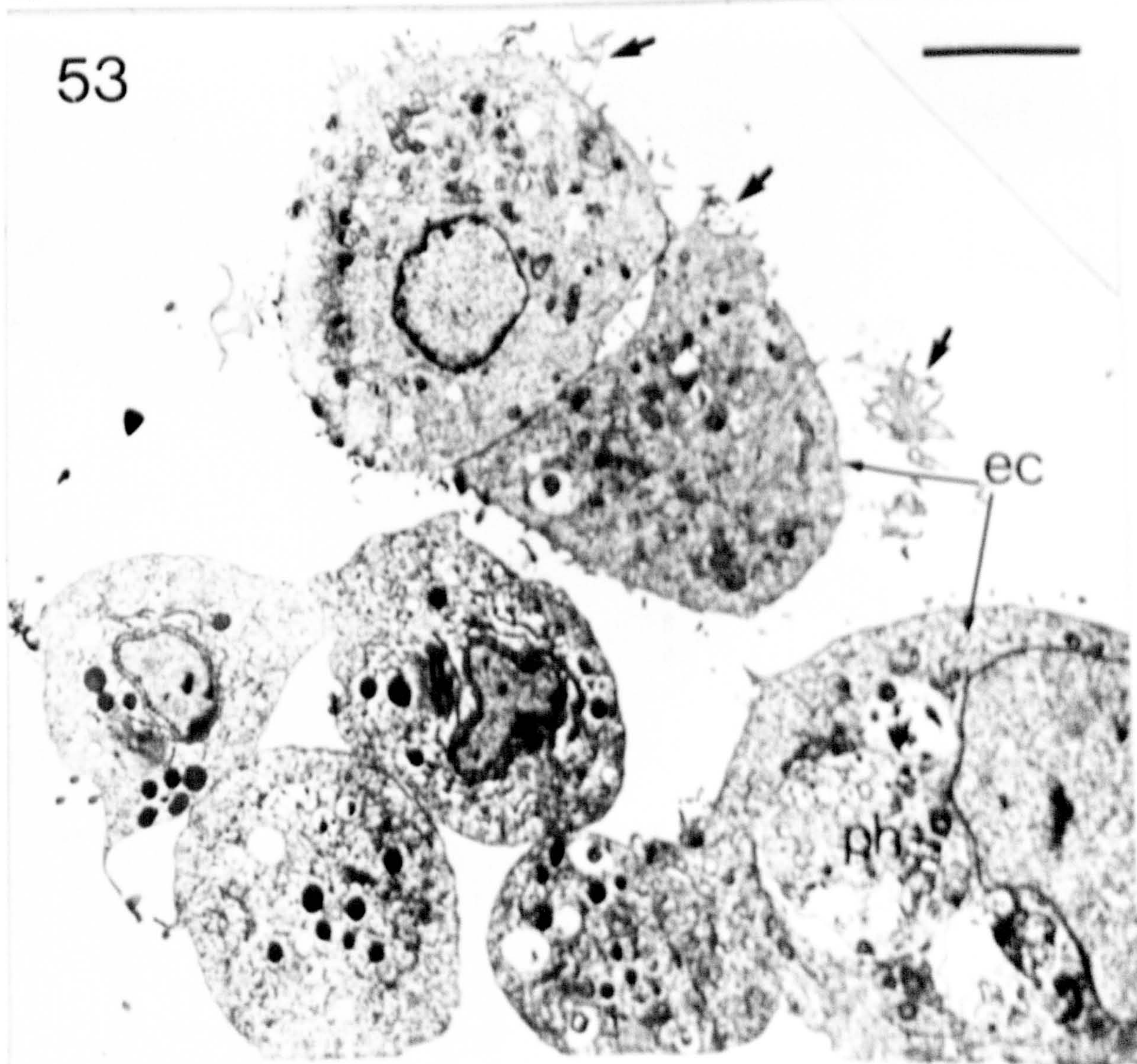
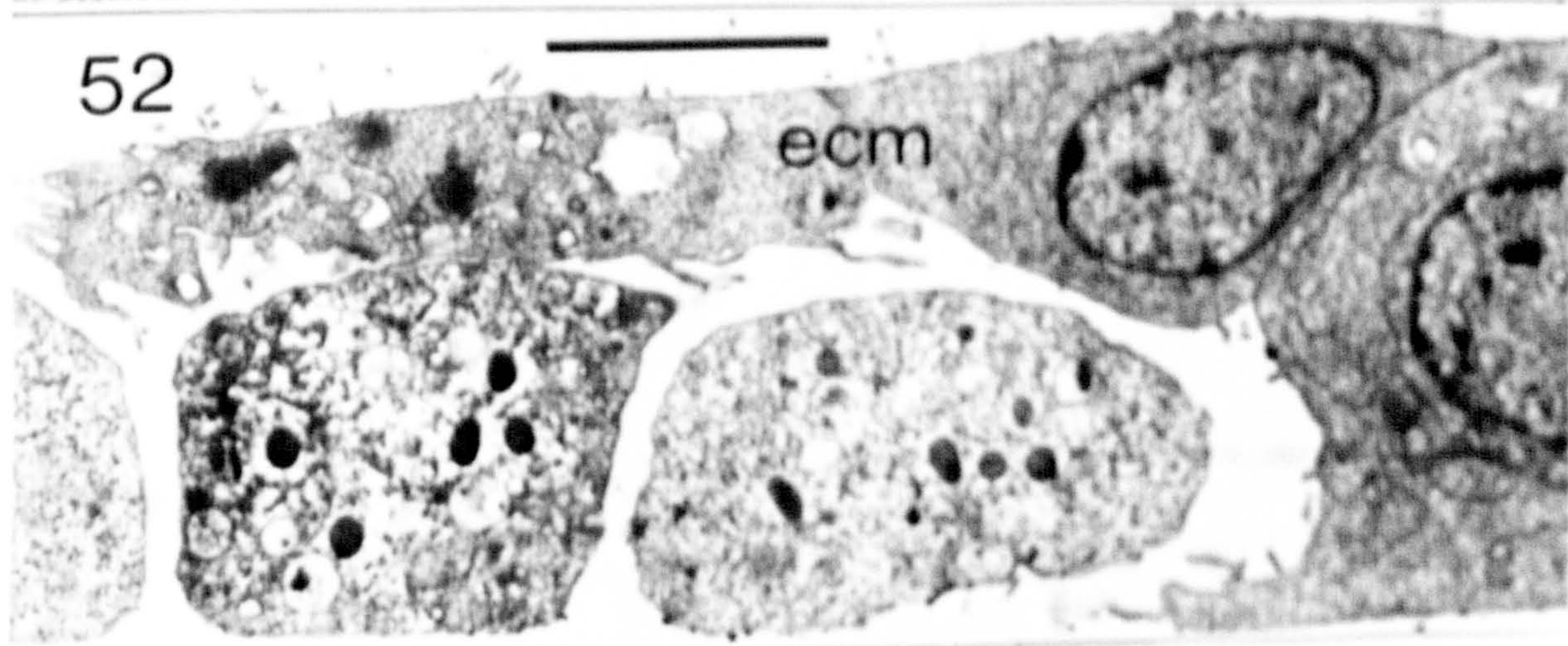




Fig. 54. VS through the edge of a lesion showing an epithelial cell which has been lifted off the substratum by a trichomonad. Note that the lifted cell and the cell adjacent to it appear morphologically normal.

Bar = 5  $\mu$ m

Fig. 55. Two T. vaginalis lying beneath the epithelial cell monolayer. Note that the anterior flagella (af) of both organisms lie in a small, i.e. less than 2  $\mu$ m wide, space between the cells and substratum; the size of the space makes it improbable that the flagella of T. vaginalis can make a significant contribution in the motility of the organism whilst lying under the monolayer of cells. Note the pseudopodia (p) of the trichomonad on the left.

Bar = 2  $\mu$ m

Fig. 56. T. vaginalis lying between the substratum and the lower surface of an epithelial cell. Note the pseudopodium (p) which makes a deep indentation in the cytoplasm of the cell and lies about 250 nm from the nuclear membrane of the epithelial cell (ec).

g = Golgi body, n = nucleus of epithelial cell.

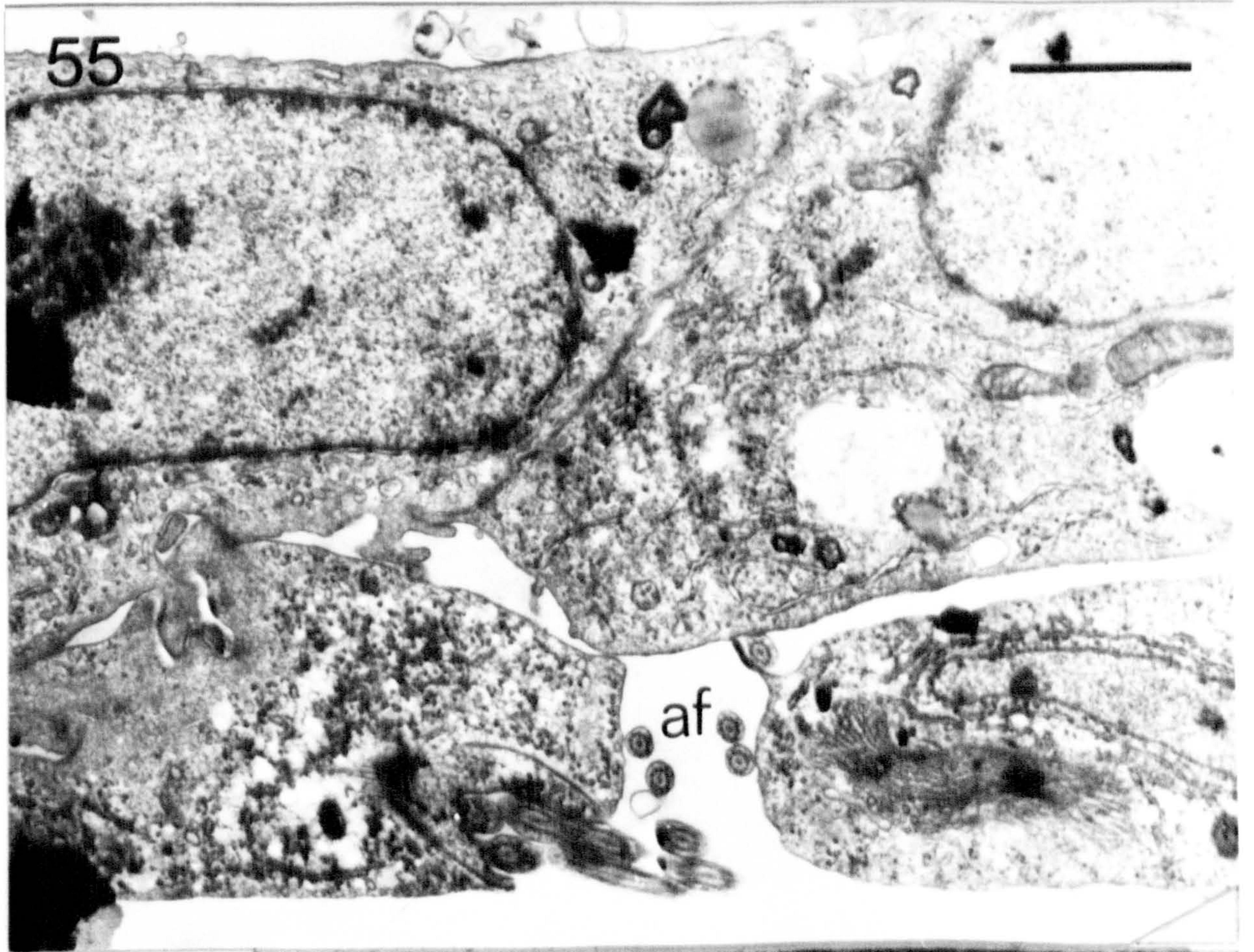
Bar = 1  $\mu$ m



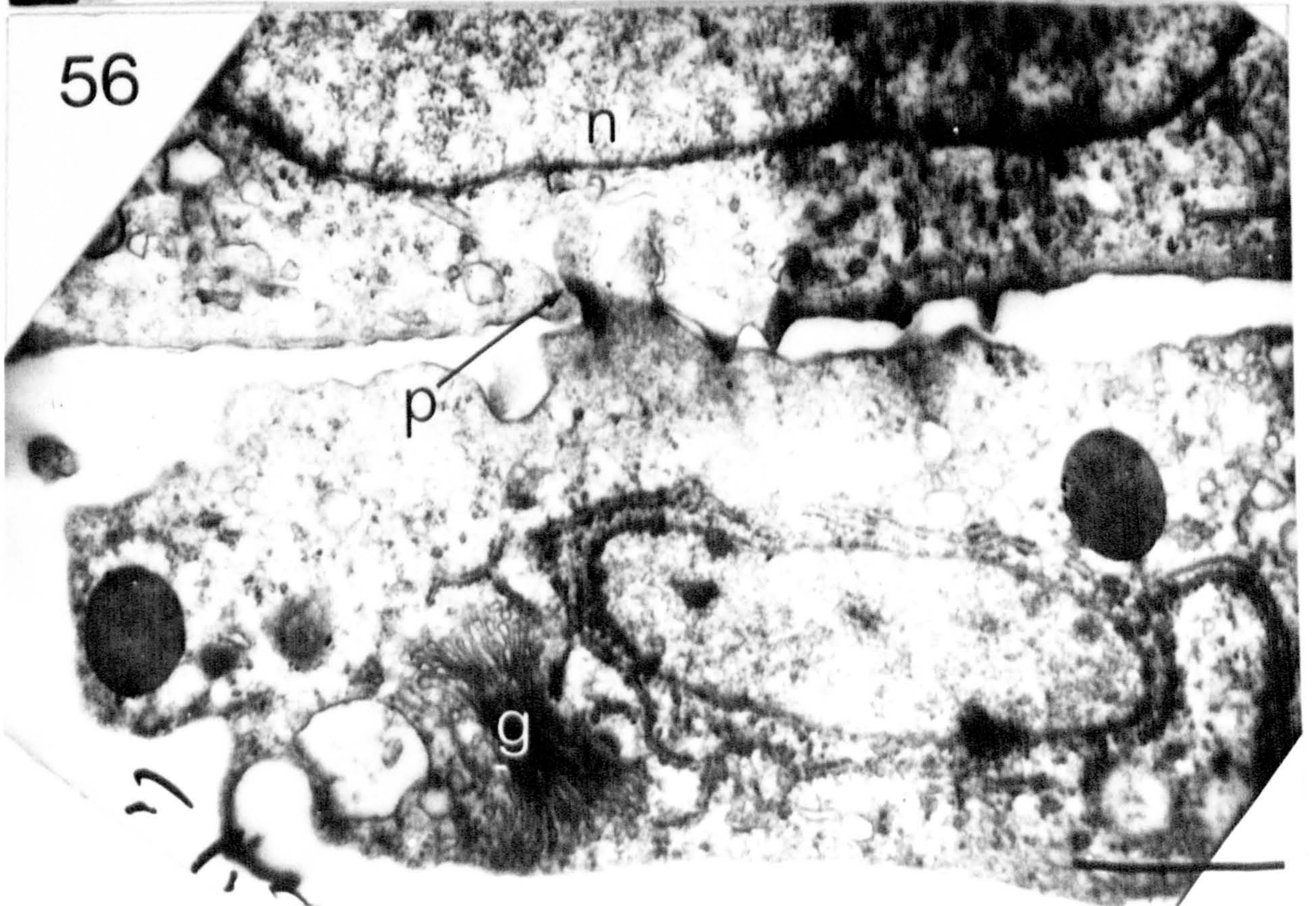
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56





Figs. 57 and 58. Type 1 contacts between T. vaginalis and epithelial cells.

Bars = 1  $\mu\text{m}$

Fig. 57. In some regions of the contact (large arrows) the parasite and cell are separated by a gap of 10 to 20 nm. Note the vesicles (small arrows) which lie close to the contacts.

Fig. 58. Region of parallel apposition of the plasma membranes of T. vaginalis and an epithelial cell. The contact is 3  $\mu\text{m}$  in length and the membranes are separated by a gap of about 20 nm. Note the vesicles (arrows) close to the contact.



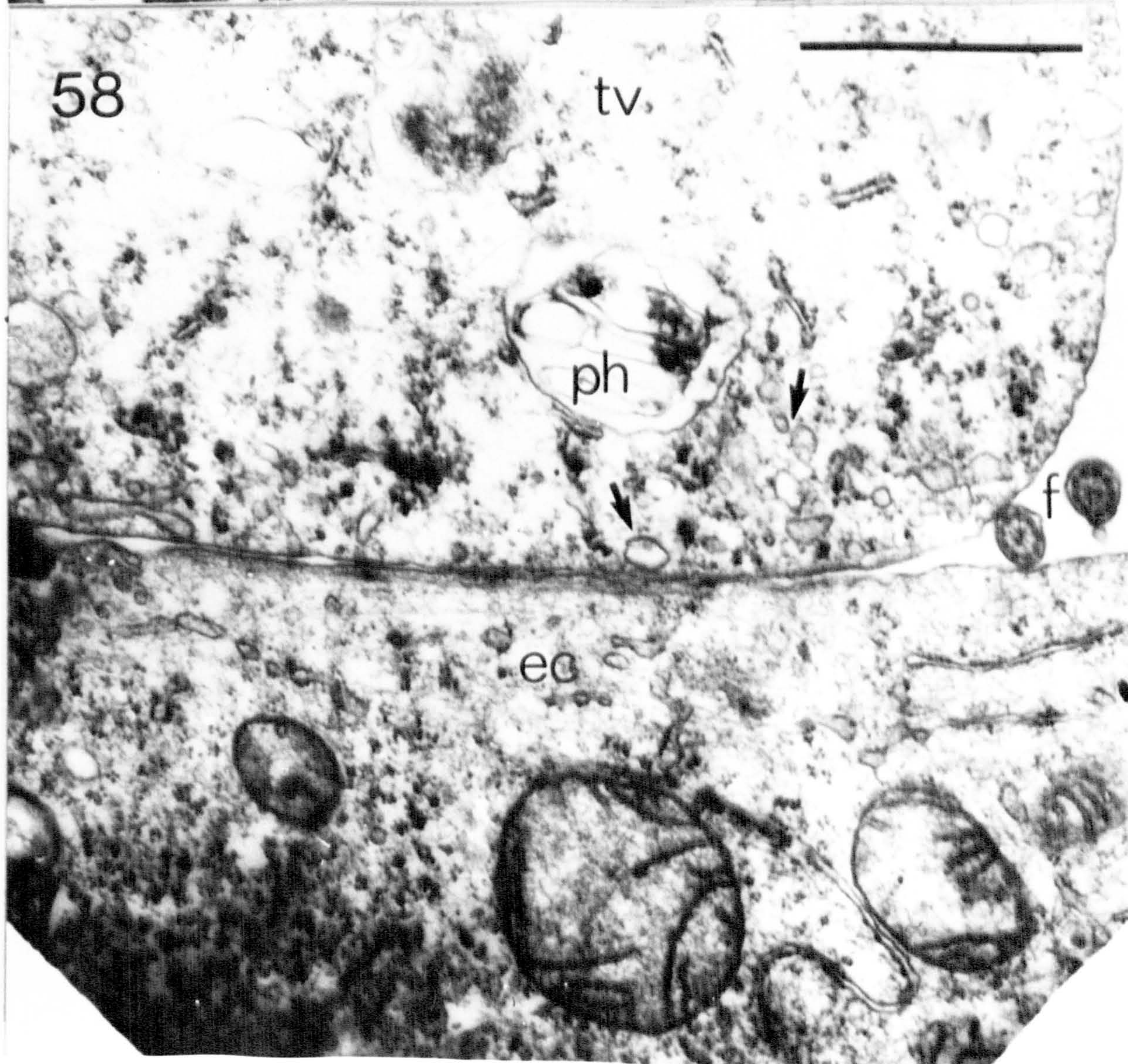
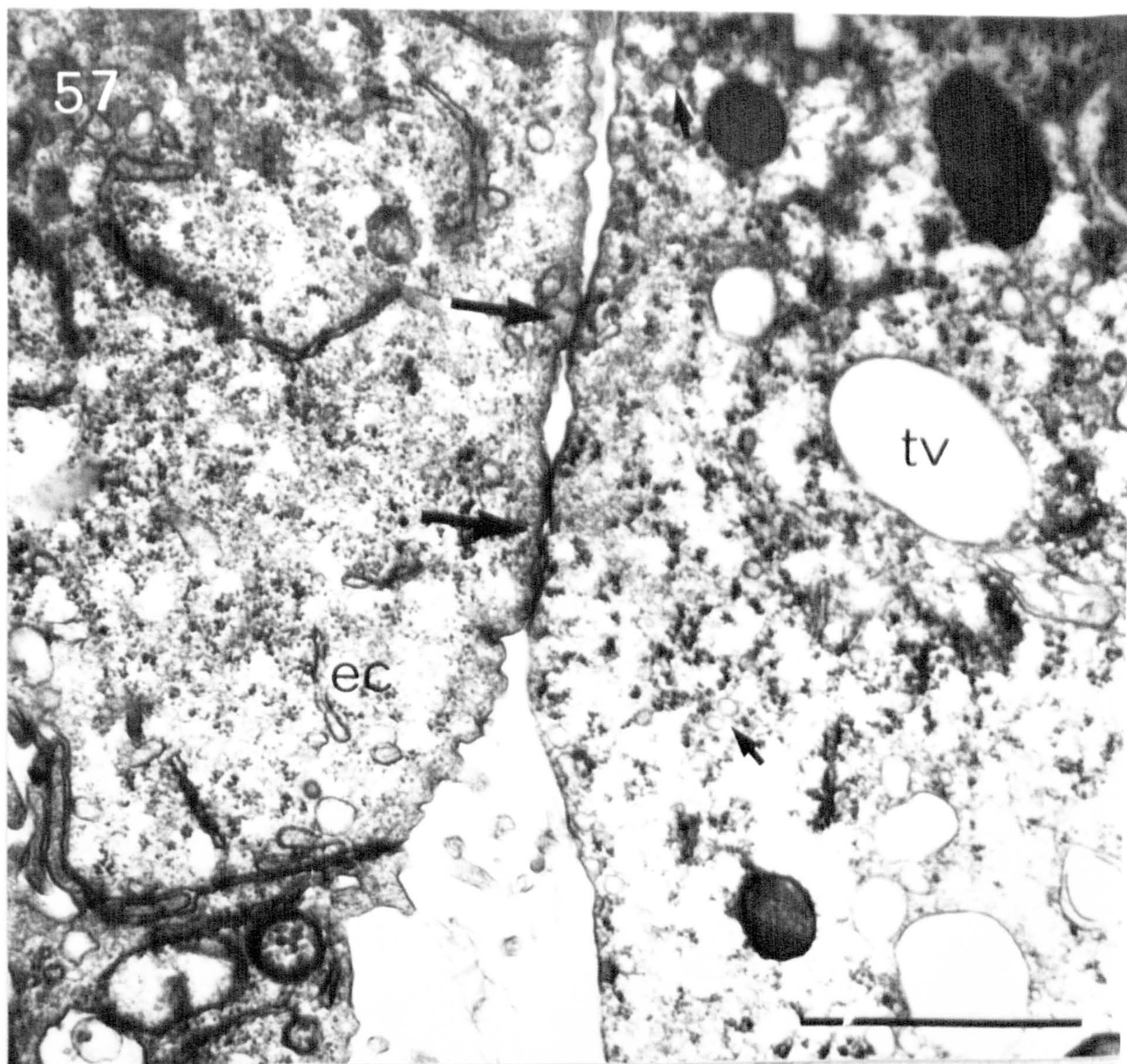




Fig. 59. Type 2 contact between T. vaginalis and an epithelial cell. The parasite has produced two pseudopodia which are nipping off a small cell process (large arrow). A cell microvillus is being phagocytosed (small arrow). Note the presence of numerous vesicles in the trichomonad (open arrows). The nucleus of the cell lies close to the region of contact with the trichomonad.

Bar = 1  $\mu$ m

Fig. 60. Type 2 contact between T. vaginalis and an epithelial cell. Two microfilament-containing pseudopodia have surrounded a finger-like process of the cell. Note the 10 nm gap between the parasite and cell (large arrow). On the right the section passes transversely through several cell processes (small arrows) which are enclosed by the pseudopodium. Note the bundle of microfilaments (mf) in the pseudopodium.

Bar = 1  $\mu$ m



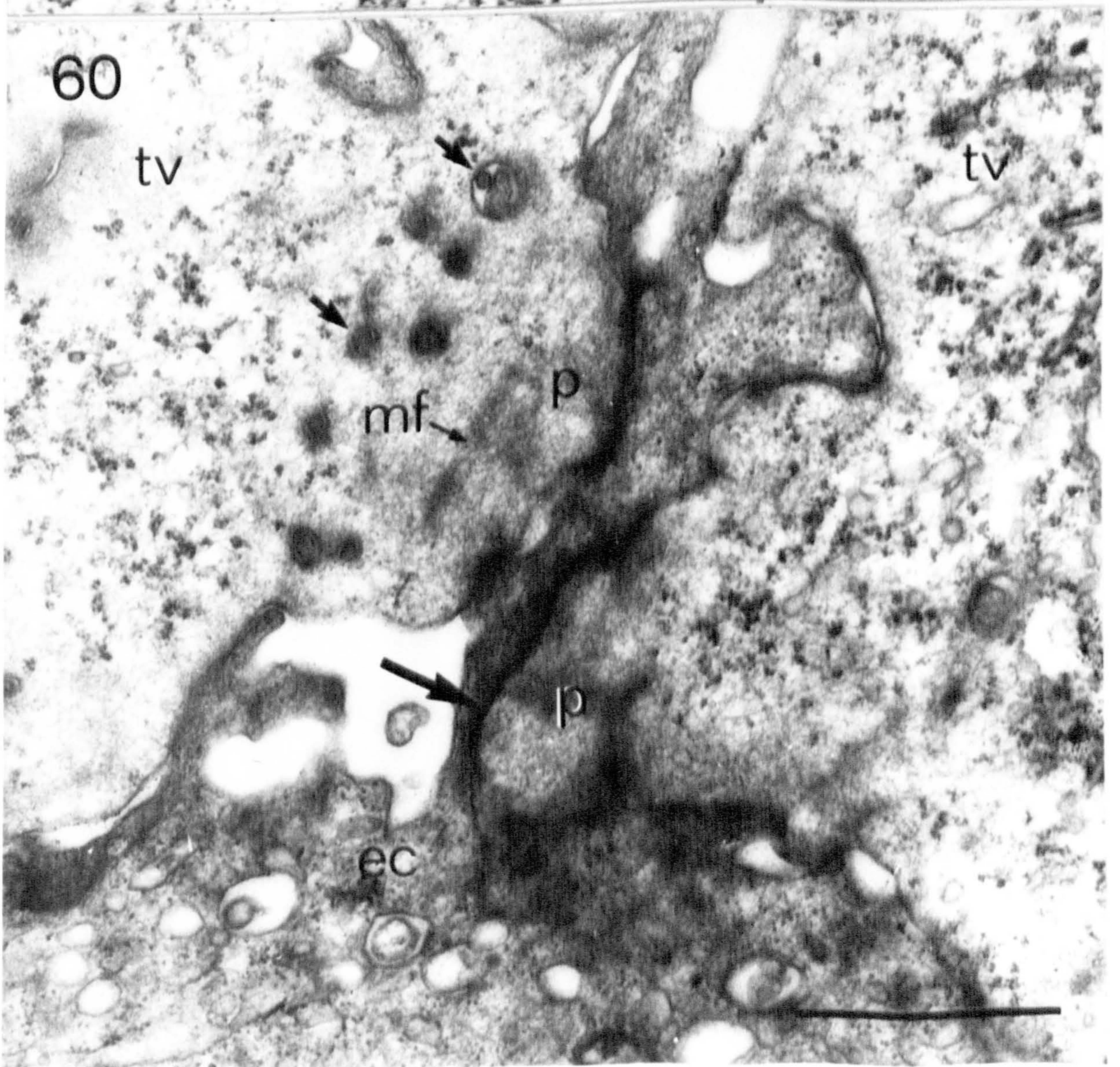
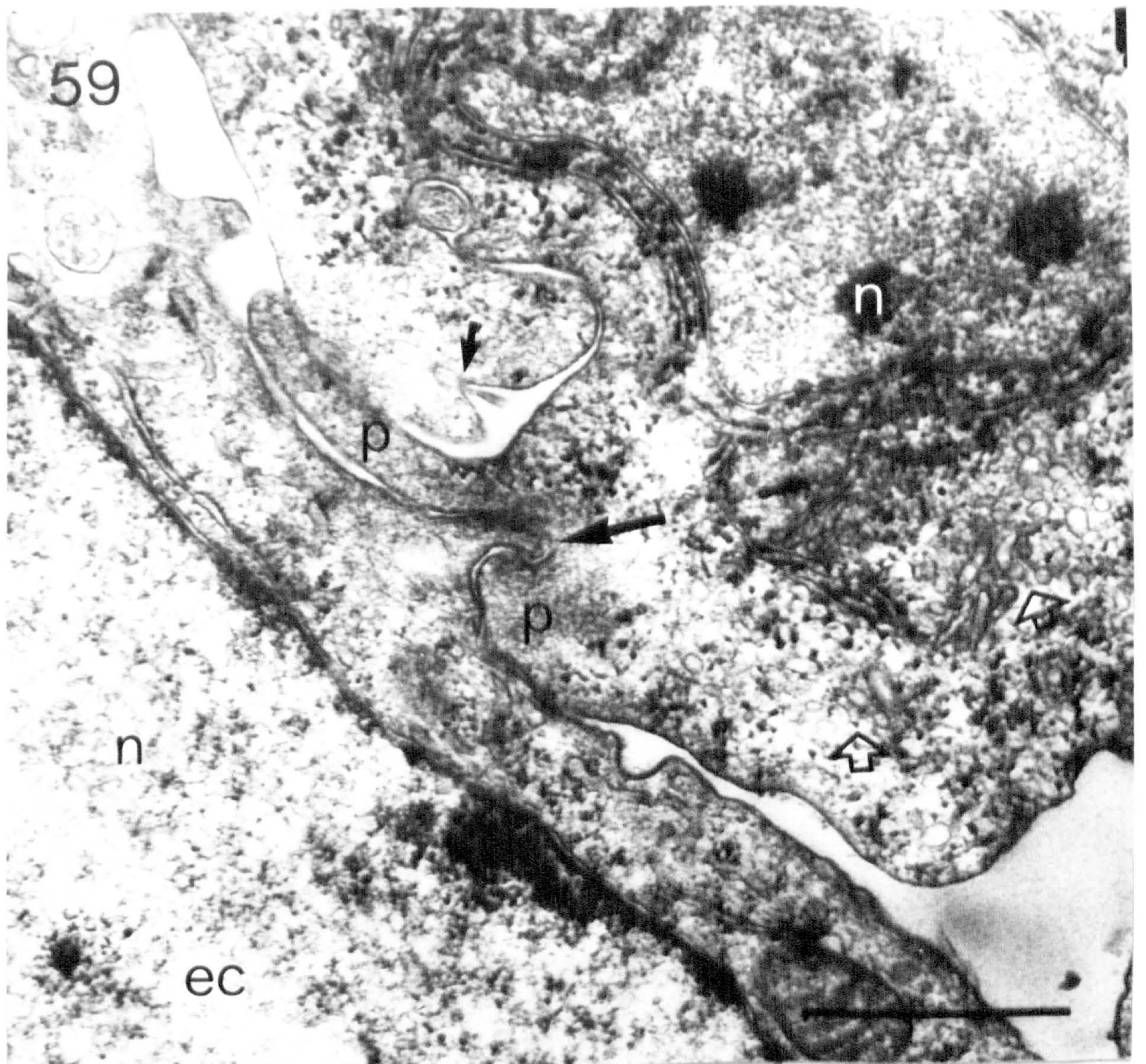
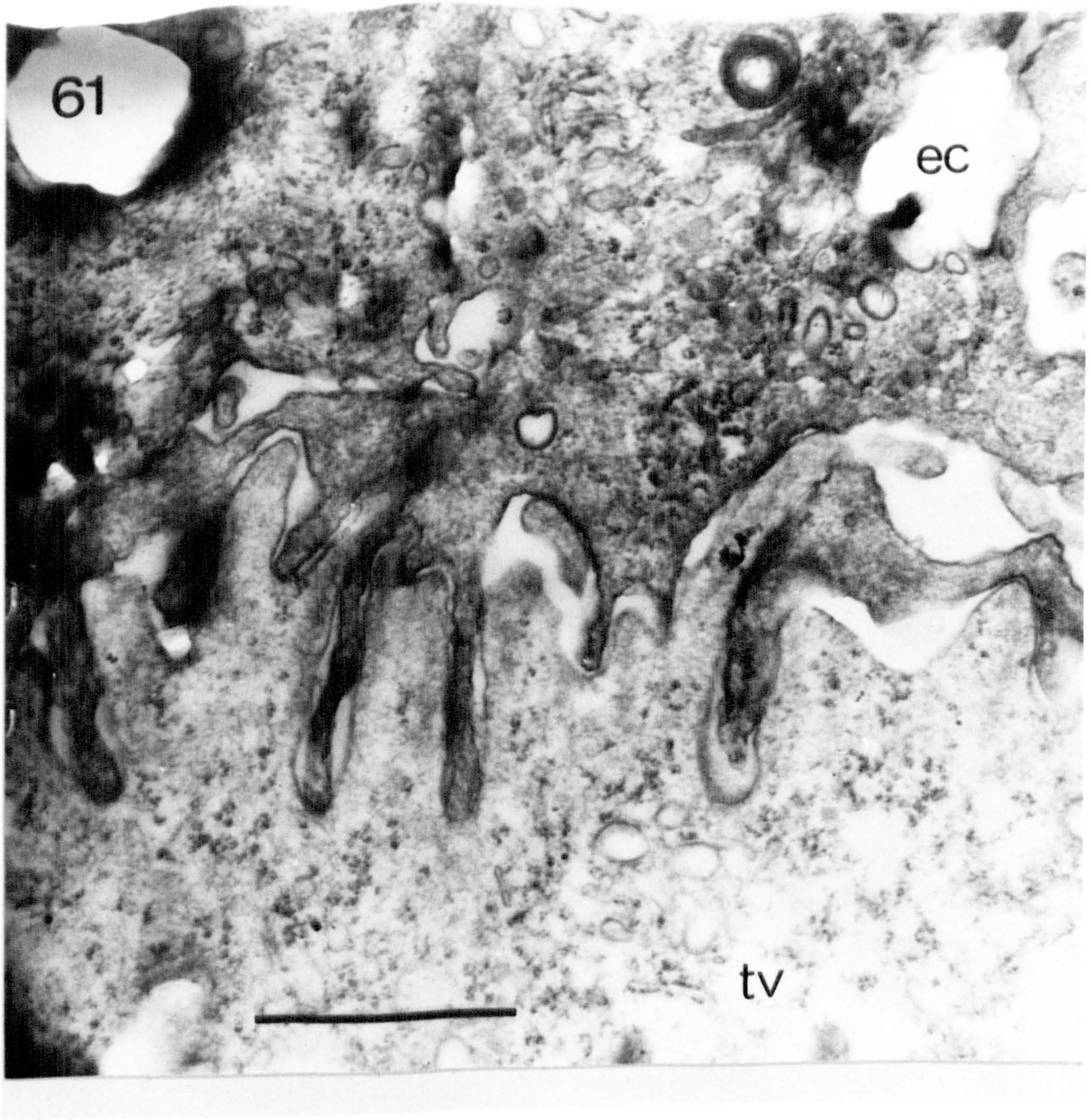




Fig. 61. Type 2 contact between T. vaginalis and an epithelial cell showing interdigitation of finger-like processes of the cell and parasite. This section passes longitudinally through a contact which is similar to that sectioned transversely in Fig. 60.

Bar = 1  $\mu$ m







Figs. 62 to 64. Type 2 contact between T. vaginalis and an epithelial cell showing the complex interdigitation of pseudopodia with cell processes.

Fig. 62. The pseudopodia of the trichomonad contain a meshwork of microfilaments which are concentrated in the regions of contact with the cell. Where the section has passed perpendicularly through the points of closest contact, the apposed membranes of T. vaginalis and the cell are clearly resolved (small arrows); but where the section passes obliquely through a contact (large arrow) the cytoplasm of trichomonad and cell misleadingly appear to be continuous. Note the similarity in appearance of the microfilament meshworks of T. vaginalis and cell (mf).

Bar = 0.5  $\mu$ m

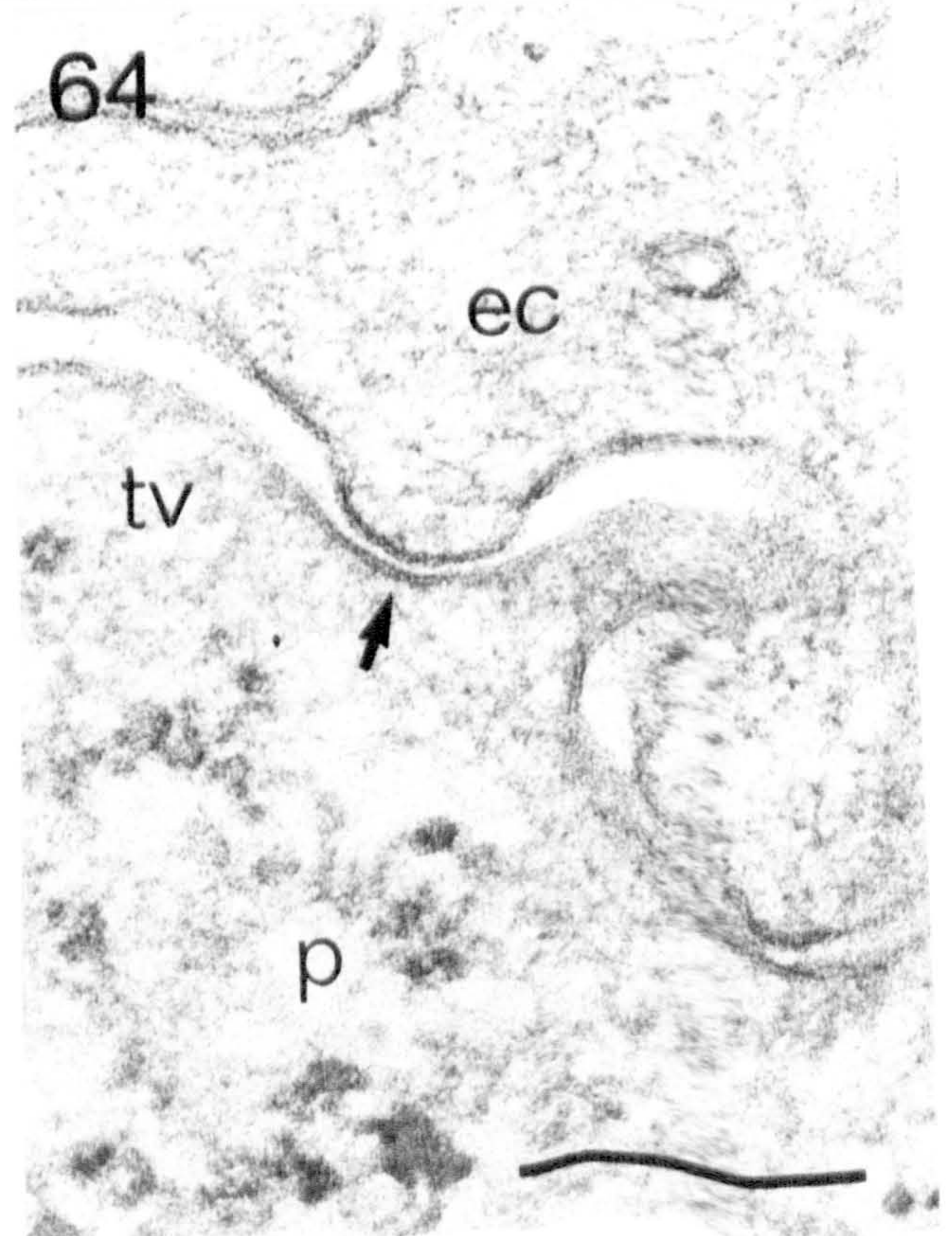
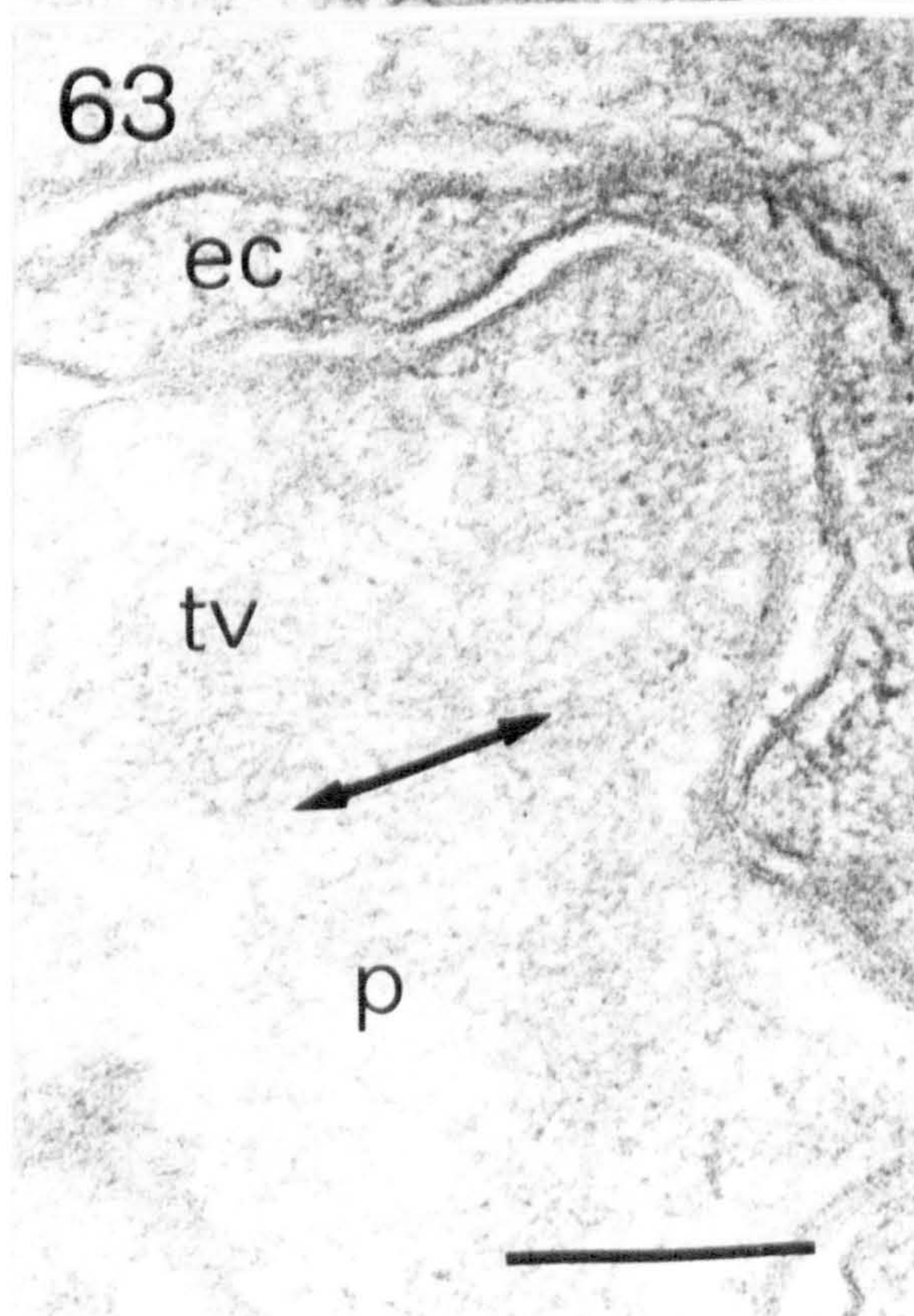
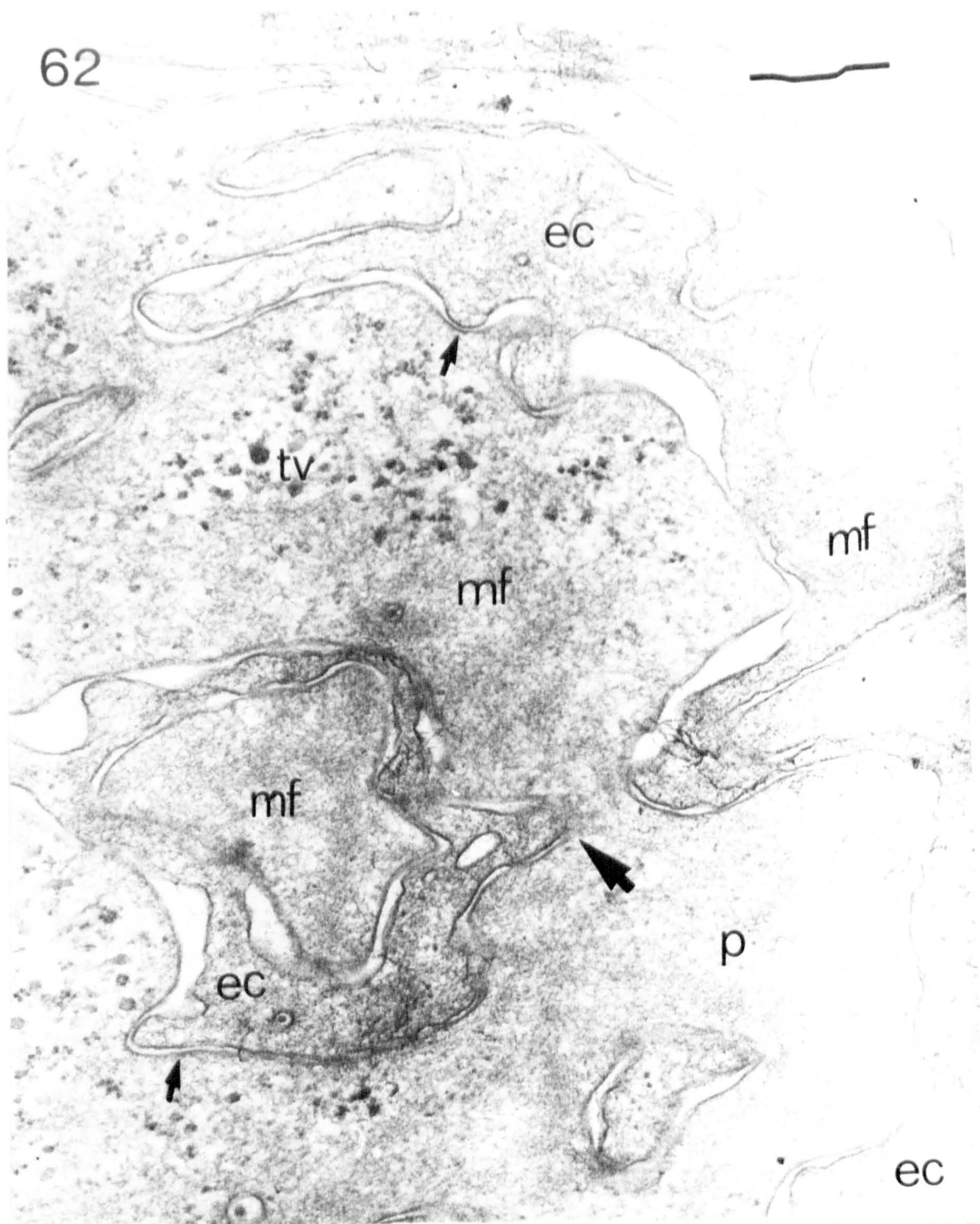
Fig. 63. Detail of Fig. 62 at higher magnification showing the 5 to 10 nm diameter microfilaments in a pseudopodium of T. vaginalis. Note the orientation of the filaments (indicated by the arrow) towards the tip of the pseudopodium.

Bar = 200 nm

Fig. 64. Detail of Fig. 62 at higher magnification showing a point of closest apposition of the membranes of T. vaginalis and epithelial cell (arrow). The trilaminar structure of both membranes is clearly resolved and there is a gap of 6 nm between them which does not contain any electron-dense material.

Bar = 200 nm







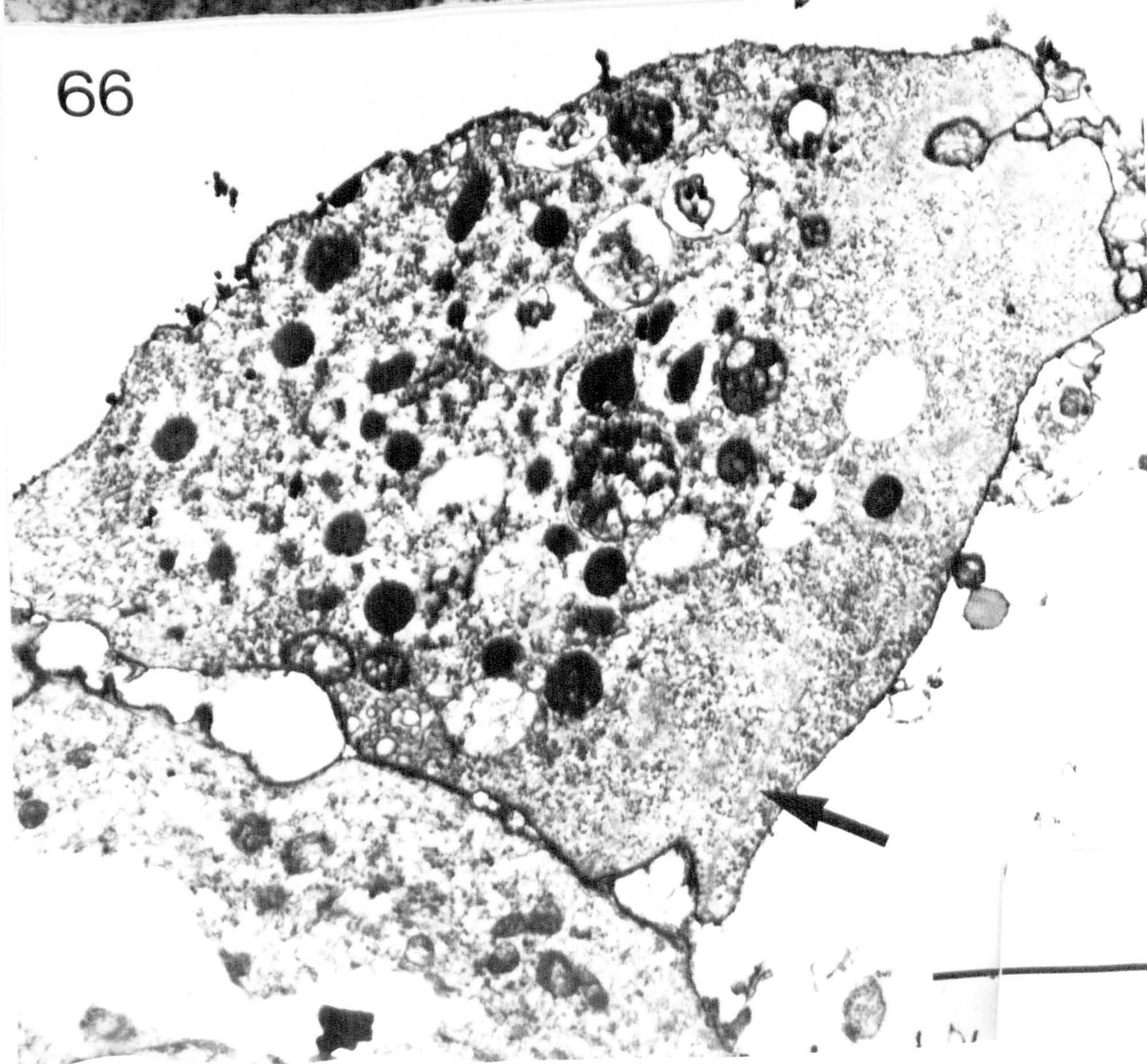
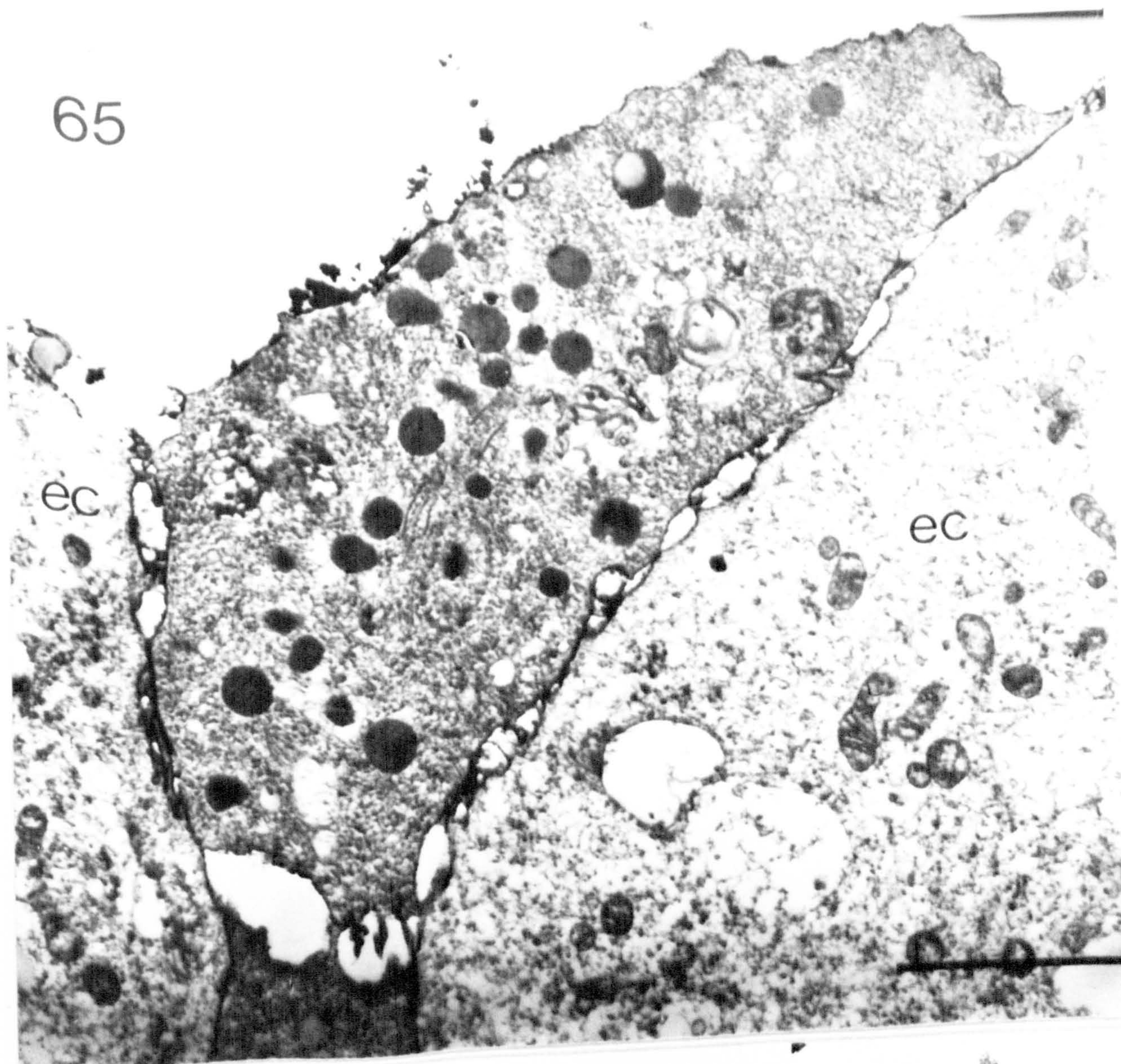
Figs. 65 and 66. T. vaginalis-infected epithelial cell cultures stained with ruthenium red (RR). The cultures were not washed before staining. Type 1 contacts are seen between the parasites and the cells.

Bars = 3  $\mu$ m

Fig. 65. A trichomonad adhering to two epithelial cells. Note the uniform electron-dense staining of the plasma membranes of both the parasite and cells. The cytoplasm of the cells appears rarified.

Fig. 66. Uniform staining of the plasma membrane of T. vaginalis. Note the microfilament bundle (arrow) within the cytoplasm of the parasite.







Figs. 67 and 68. High magnification views of the regions of contact of T. vaginalis (tv) with epithelial cells (ec) in cultures stained with ruthenium red.

Bars = 500 nm

Fig. 67. The plasma membranes of both trichomonad and cell are covered with a 15 nm thick electron-dense ruthenium red positive layer. Note that the stain has penetrated between even the closest points of contact (arrow). The cytoplasm of the epithelial cell is much less electron-dense than normal. There is some RR stained material lying within a phagosome (ph) and within a stack of membranes (open arrow) indicating that these structures must have an opening to the surface of the organism since RR does not penetrate the plasma membranes of cells.

Fig. 68. 15 nm thick layers of RR positive material on the plasma membranes of trichomonad and epithelial cell (arrows). Note that the stain penetrates between the closest points of contact. The cytoplasm of the epithelial cell is rarefied due to the condensation of particulate material into small amorphous aggregates.

Fig. 69. Ruthenium red positive staining of the plasma membrane of T. vaginalis fixed without prior washing. There is an irregular 40 nm thick surface layer to which larger particles of RR stained material are adhering. Two pinocytotic vesicles (v) lie within the cytoplasm. Both vesicles have an internal 40 nm thick surface coat; that of the lefthand vesicle has stained with RR, indicating that the vesicle has an opening to the exterior out of the plane of section.

Bar = 500 nm



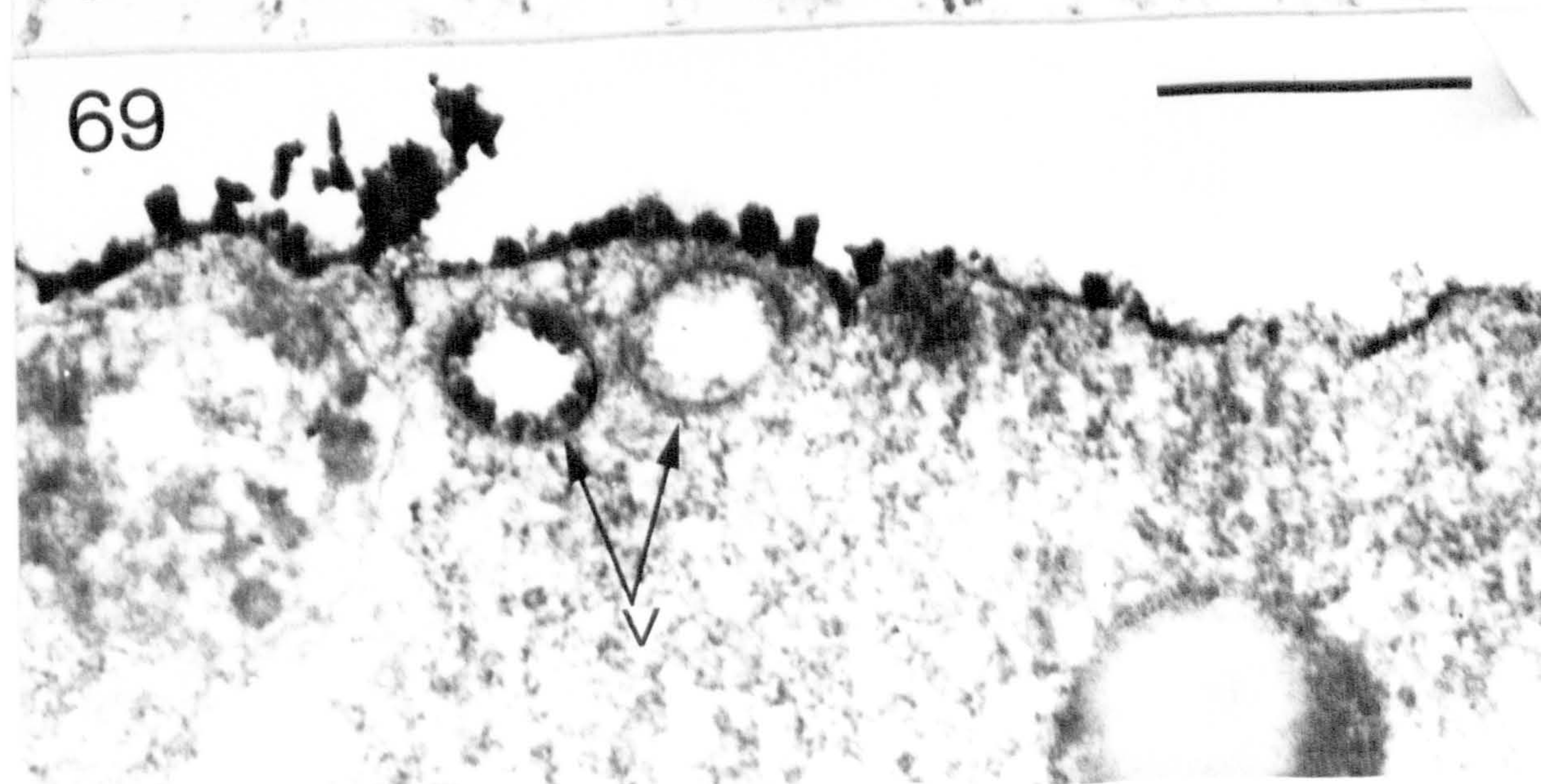
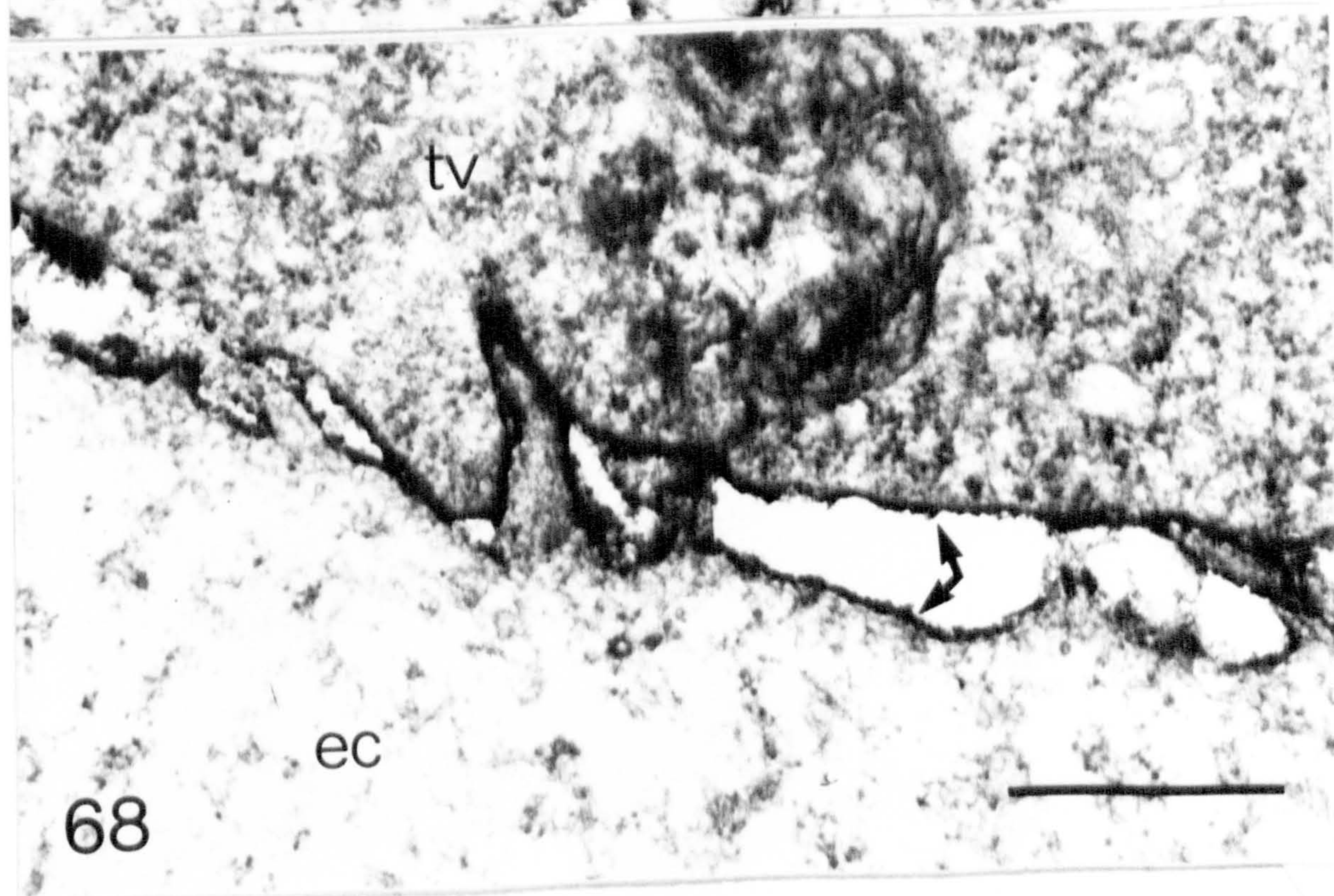
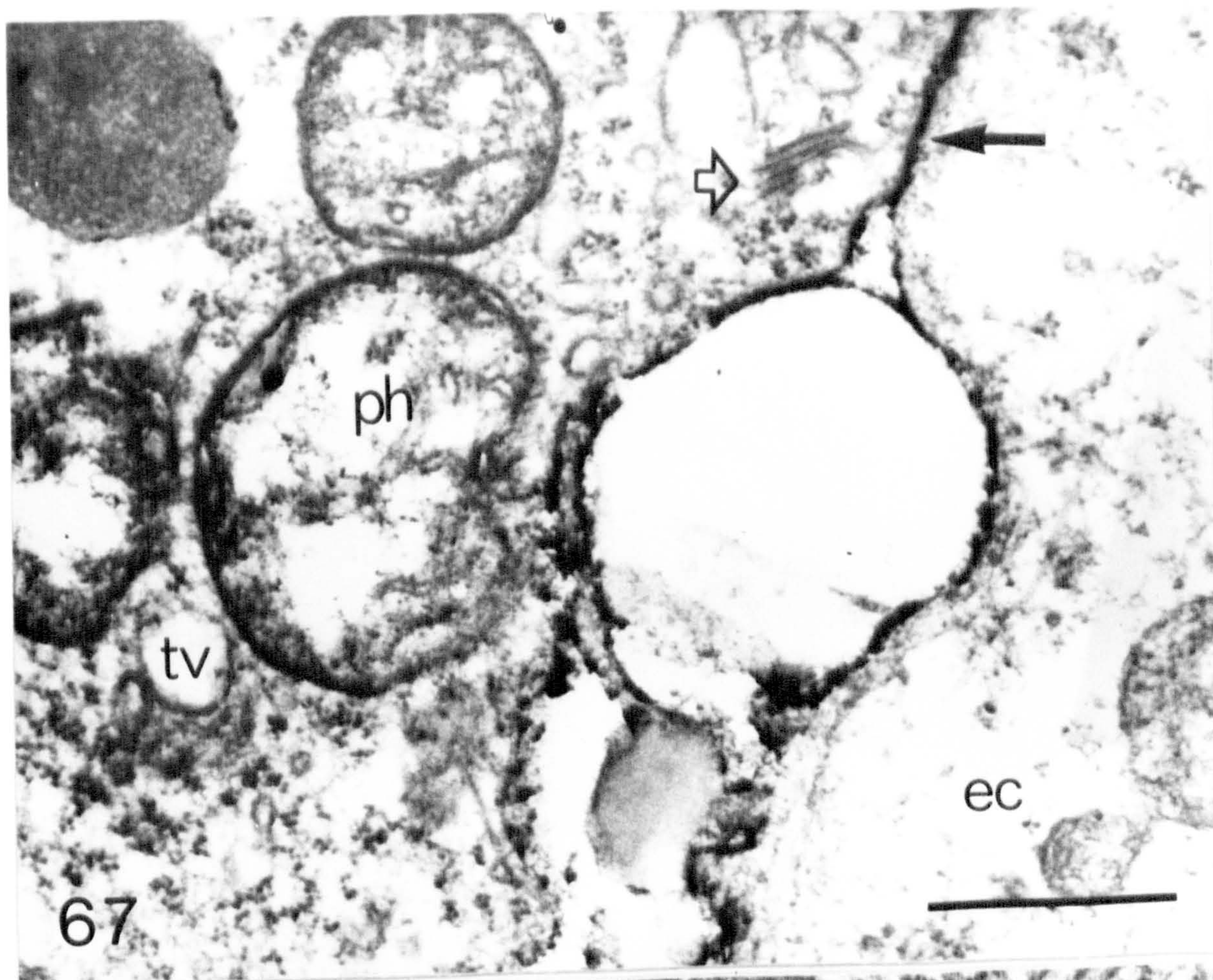




Fig. 70. T. vaginalis stained with ruthenium red after a thorough washing in serum-free medium. Note the greatly reduced amount of staining of the plasma membrane.

Bar = 2  $\mu$ m

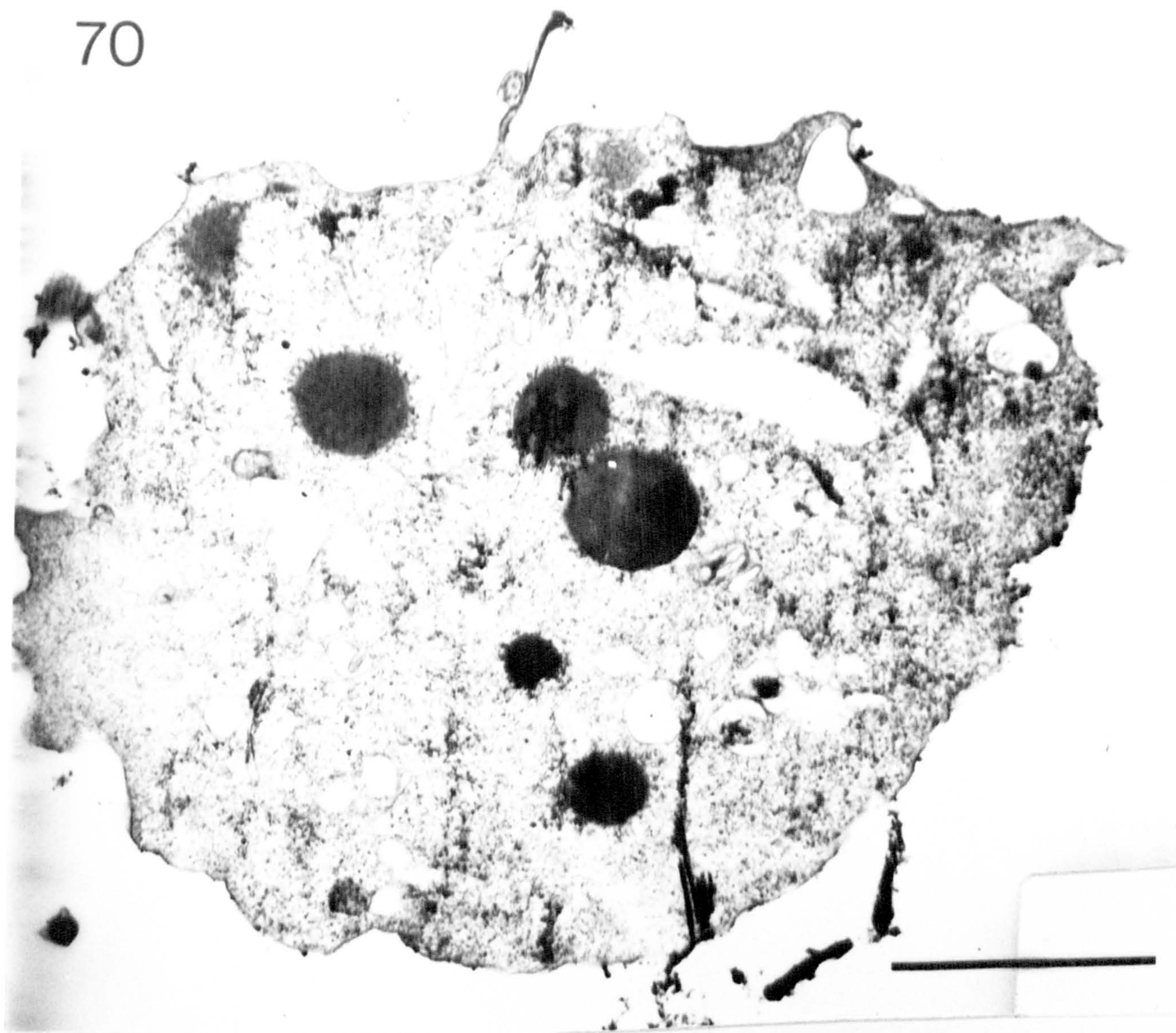
Fig. 71. T. vaginalis stained with ruthenium red after washing in serum-free medium and incubation in neuraminidase at 37°C for 30 min. There is an almost total absence of staining of the plasma membrane. Note the axostylar projection (ax) at the posterior pole of the body.

n = nucleus

Bar = 5  $\mu$ m



70



71

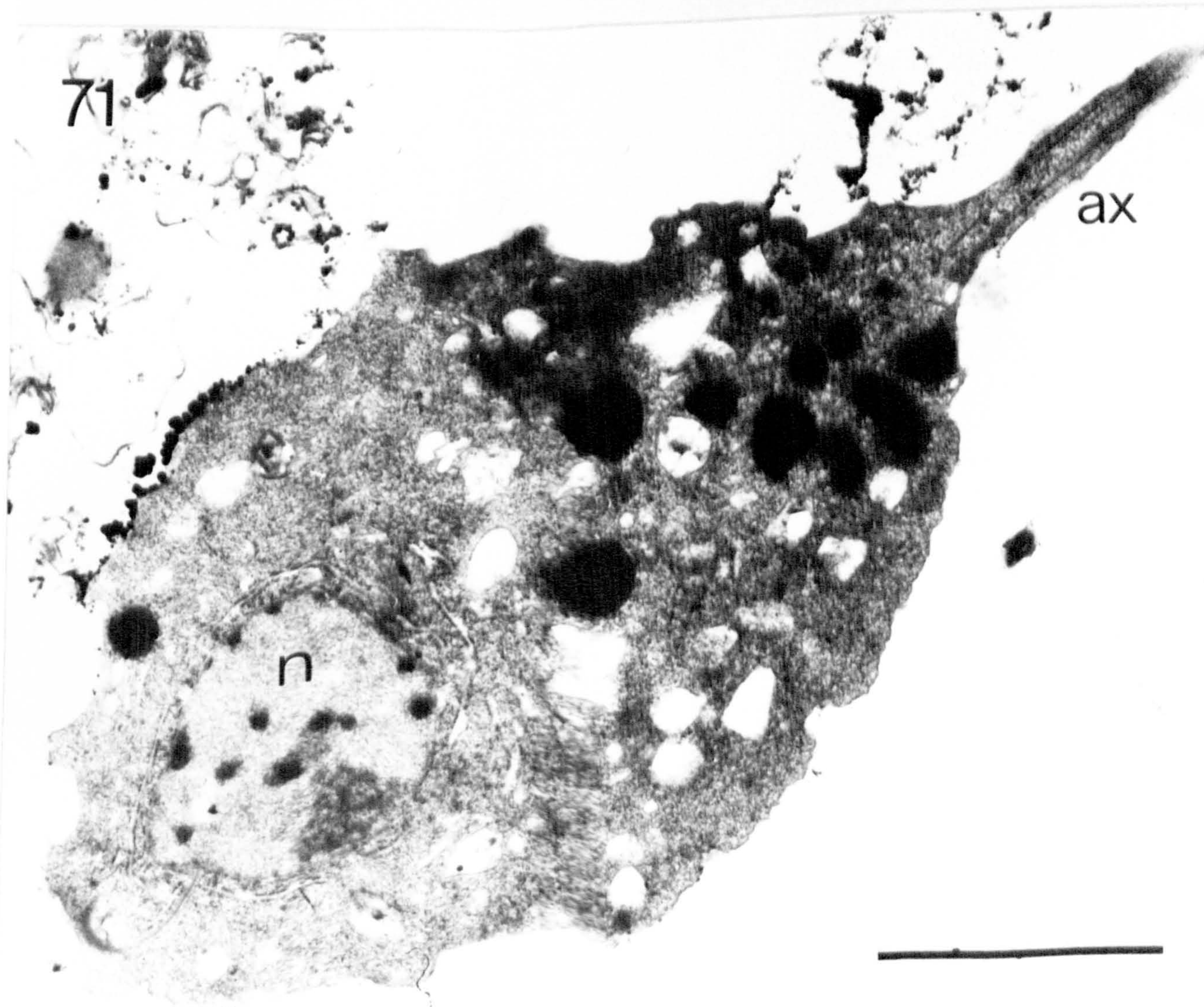




Fig. 72. Early morphological changes in epithelial cell cultures infected for 6 h with T. vaginalis. Two trichomonads lie above an epithelial cell. The cell projects much further into the medium than normal and its cytoplasm contains many small vacuoles (va) and mitochondria (m) which have lost their cristae.

Bar = 5  $\mu$ m

Fig. 73. A type 1 contact between T. vaginalis and an epithelial cell. Note the close, average 20 nm, gap between the apposed membranes (small arrow). The epithelial cell contains three large vacuoles (va); the vacuolar membranes show signs of damage and disorganisation (large arrows).

Bar = 1  $\mu$ m



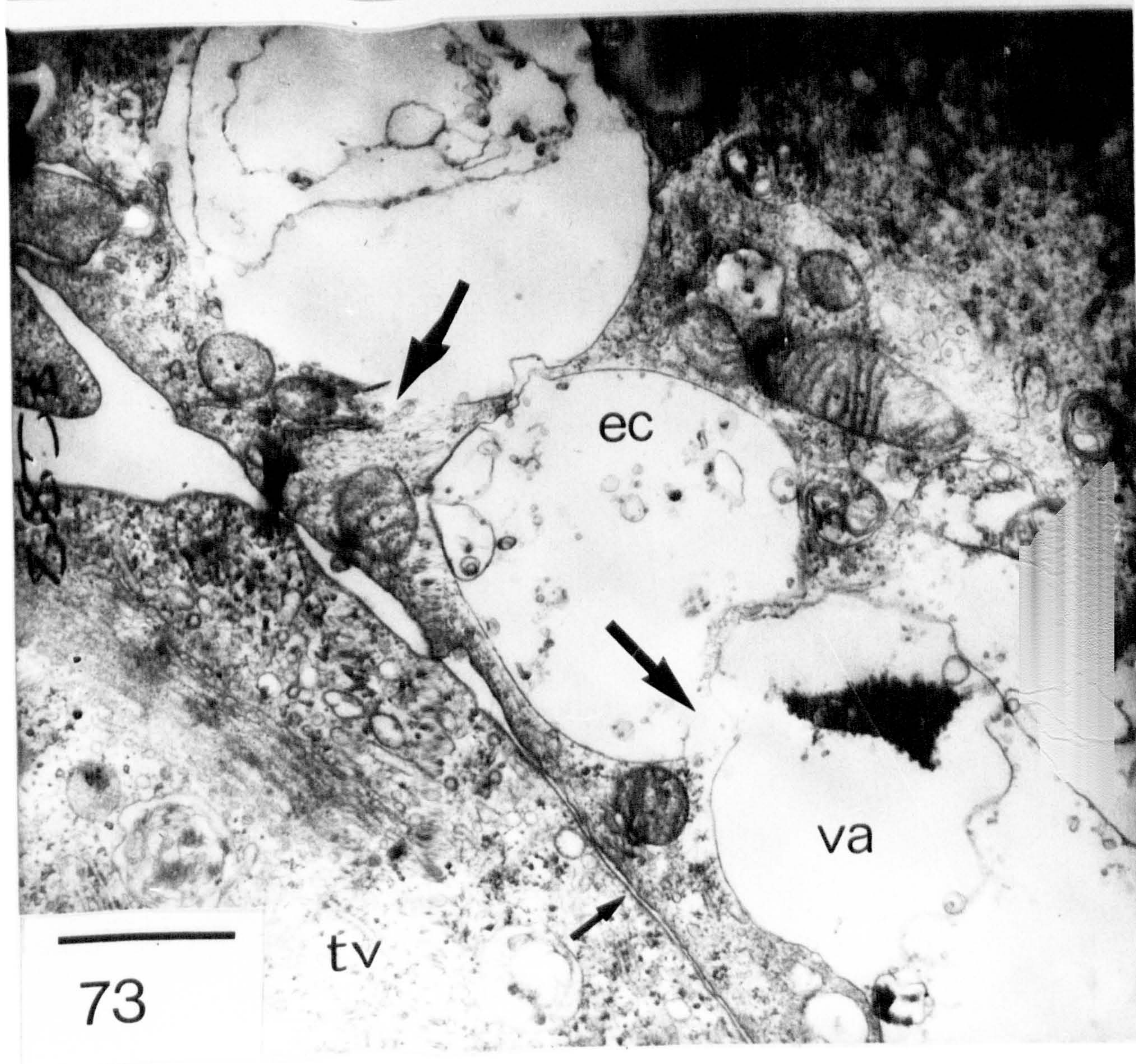
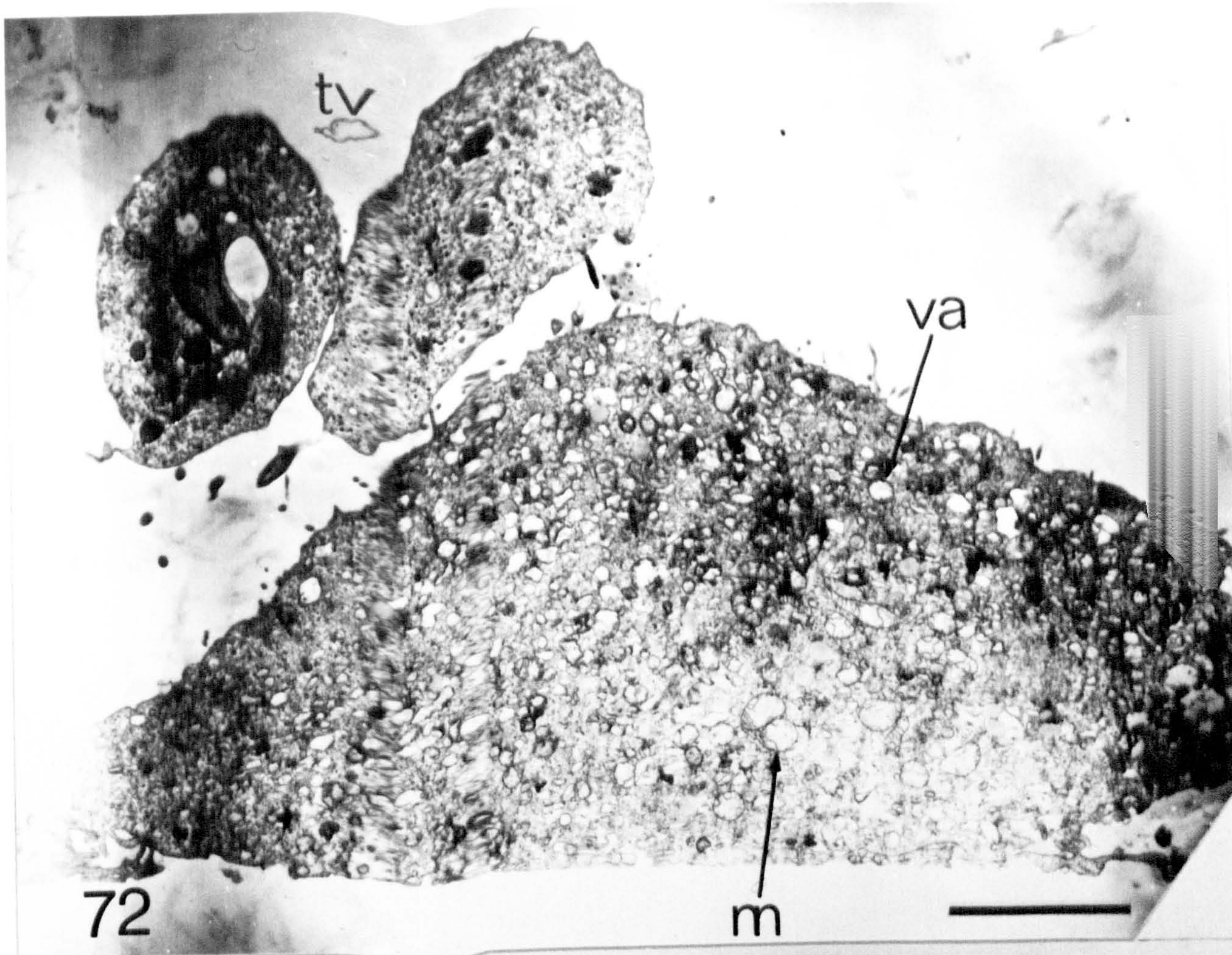




Fig. 74. Early morphological changes in epithelial cells. The mitochondria (m) of this cell, to which a trichomonad is adherent, are rounded in cross section and some have lost their cristae (arrows). Note that the trichomonad has produced pseudopodia only in the area of contact with the cell (p).

Bar = 2  $\mu$ m

Fig. 75. Lysis of epithelial cells by T. vaginalis. A group of three trichomonads have attacked a cell and lysed it. One parasite is phagocytosing the cell contents (arrow). Note that the cells in the monolayer beneath the trichomonads appear normal.

Bar = 5  $\mu$ m



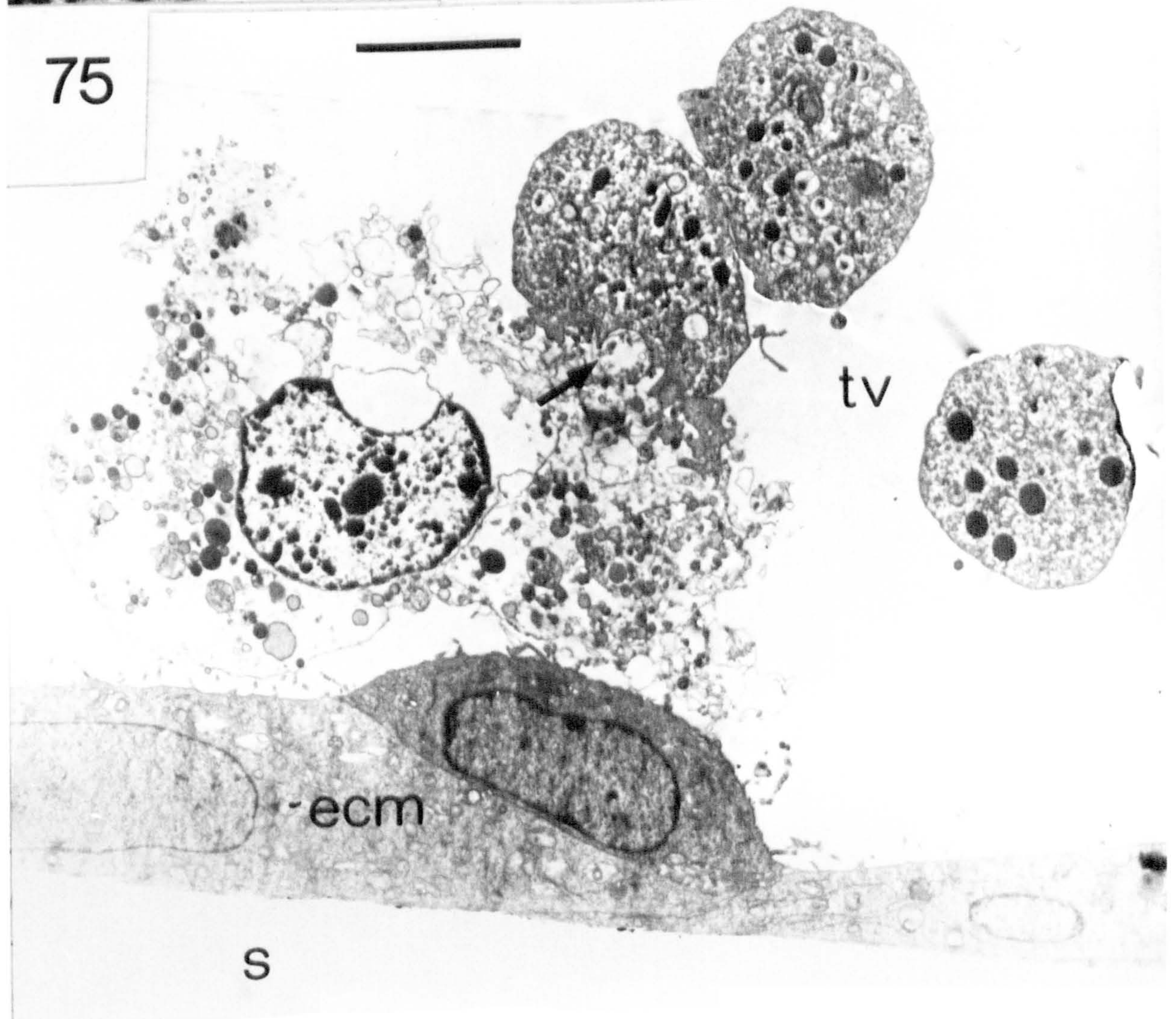
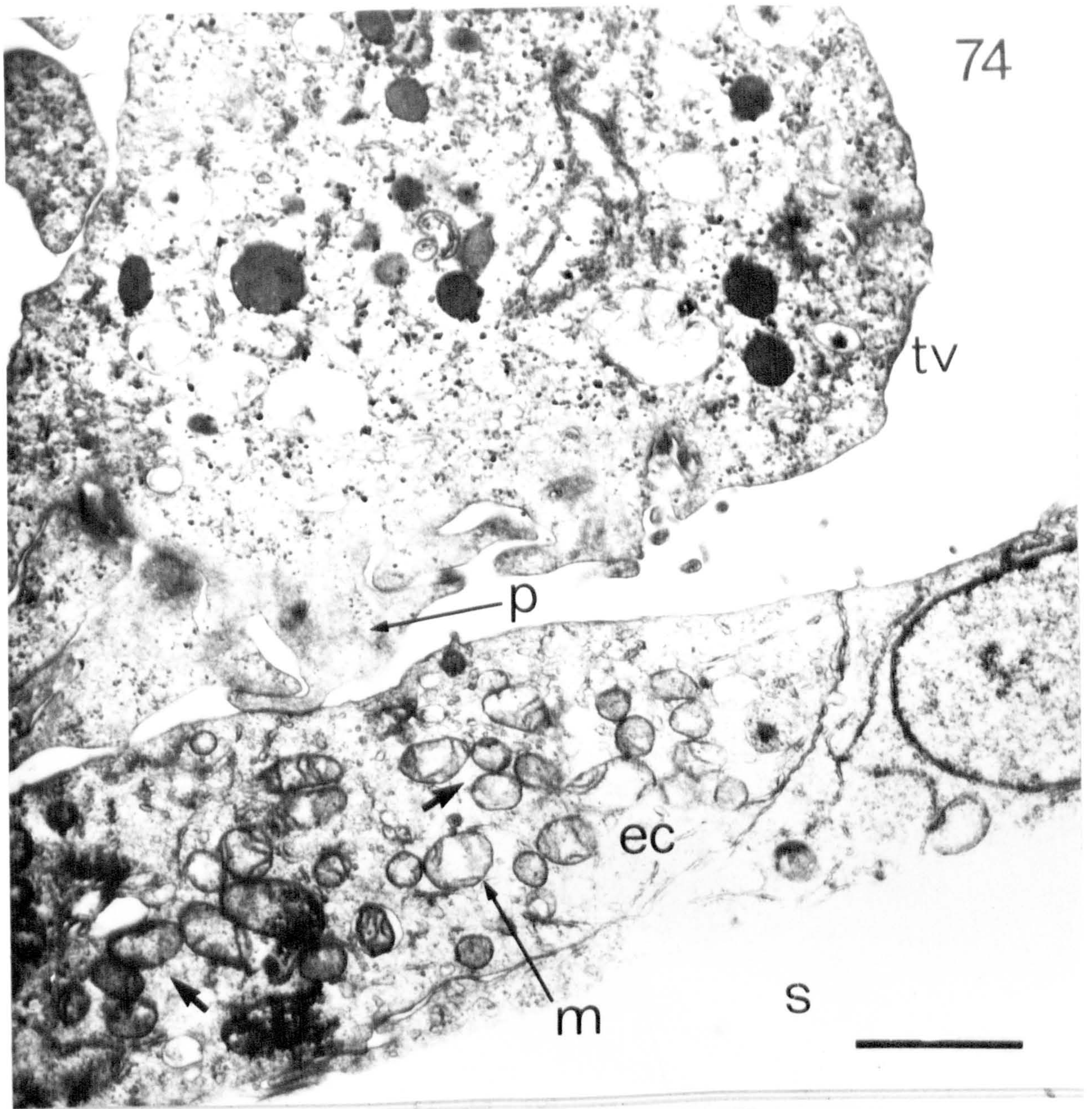




Fig. 76. Vertical section through the edge of a lesion showing two trichomonads lying beneath a lysed epithelial cell. The plasma membrane of the lysed cell is still complete on its upper surface but is lifted away from the cytoplasm (long arrow). The lysed cell is still adherent to an adjacent, unlysed cell (short arrow). The nucleus and cytoplasm of the lysed cell have lost most of their electron density (cf. the unlysed cell on the right). One trichomonad has invaded the lysed cell and is phagocytosing the contents with its pseudopodia (p).  
Bar = 5  $\mu$ m



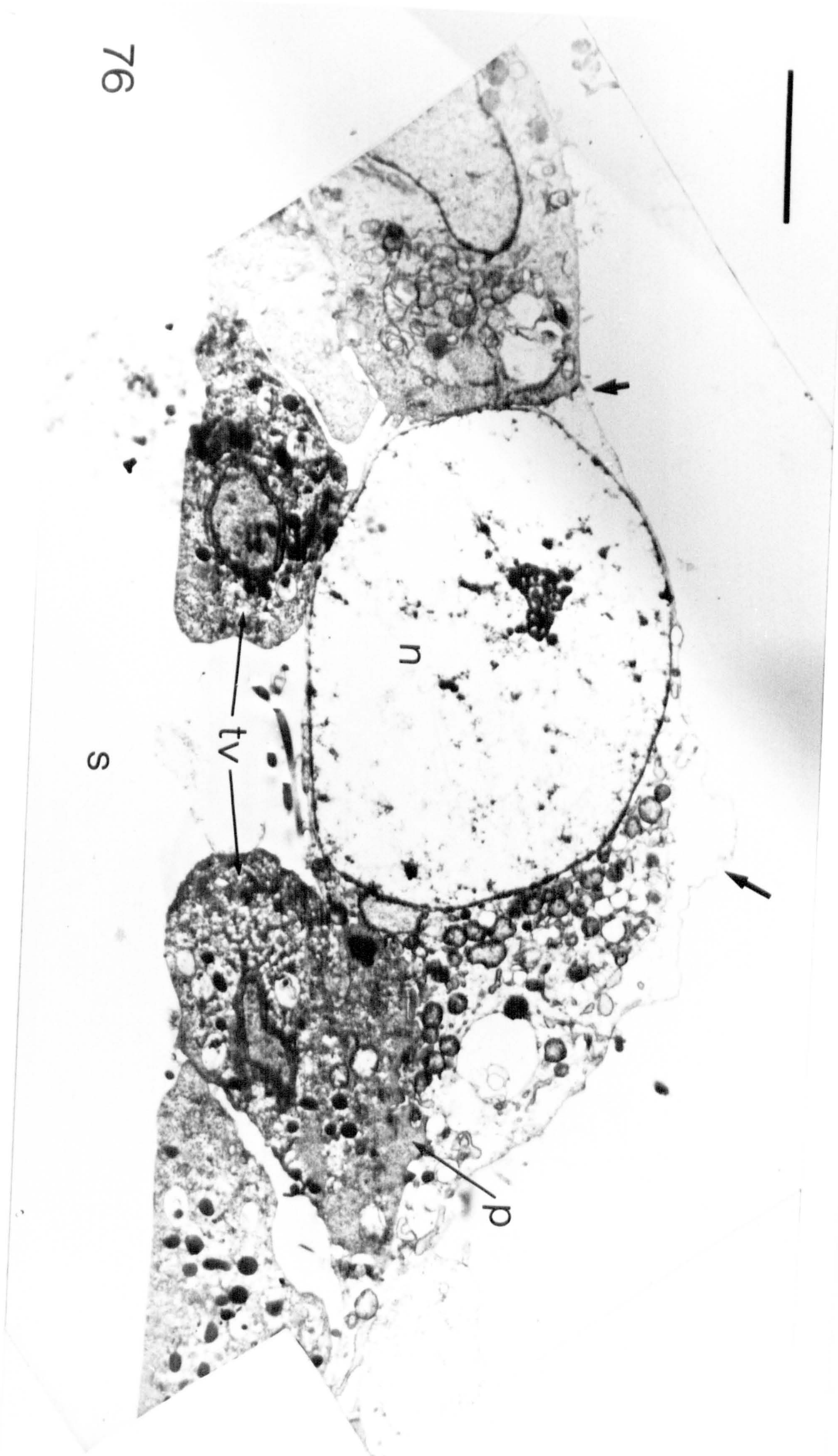
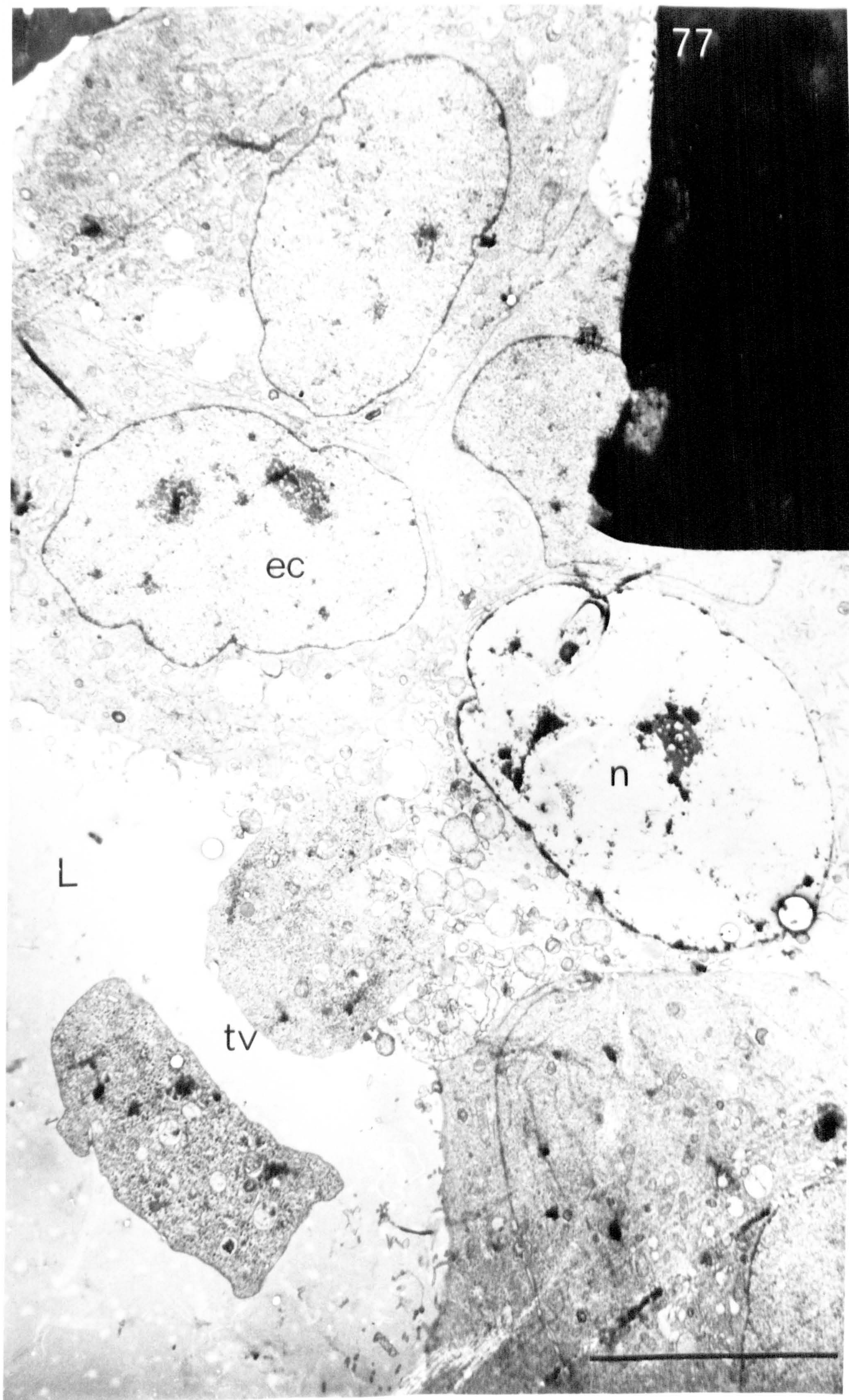




Fig. 77. Horizontal section through the edge of a lesion (L) showing a trichomonad invading and phagocytosing the contents of a lysed epithelial cell. Note that the epithelial cells adjacent to the lysed cell appear undamaged. The area of phagocytosis is shown at higher magnification in Fig. 82.

Bar = 10  $\mu$ m







Figs. 78 and 79, HS through the edge of a lesion showing T. vaginalis adhering to the side of an epithelial cell.

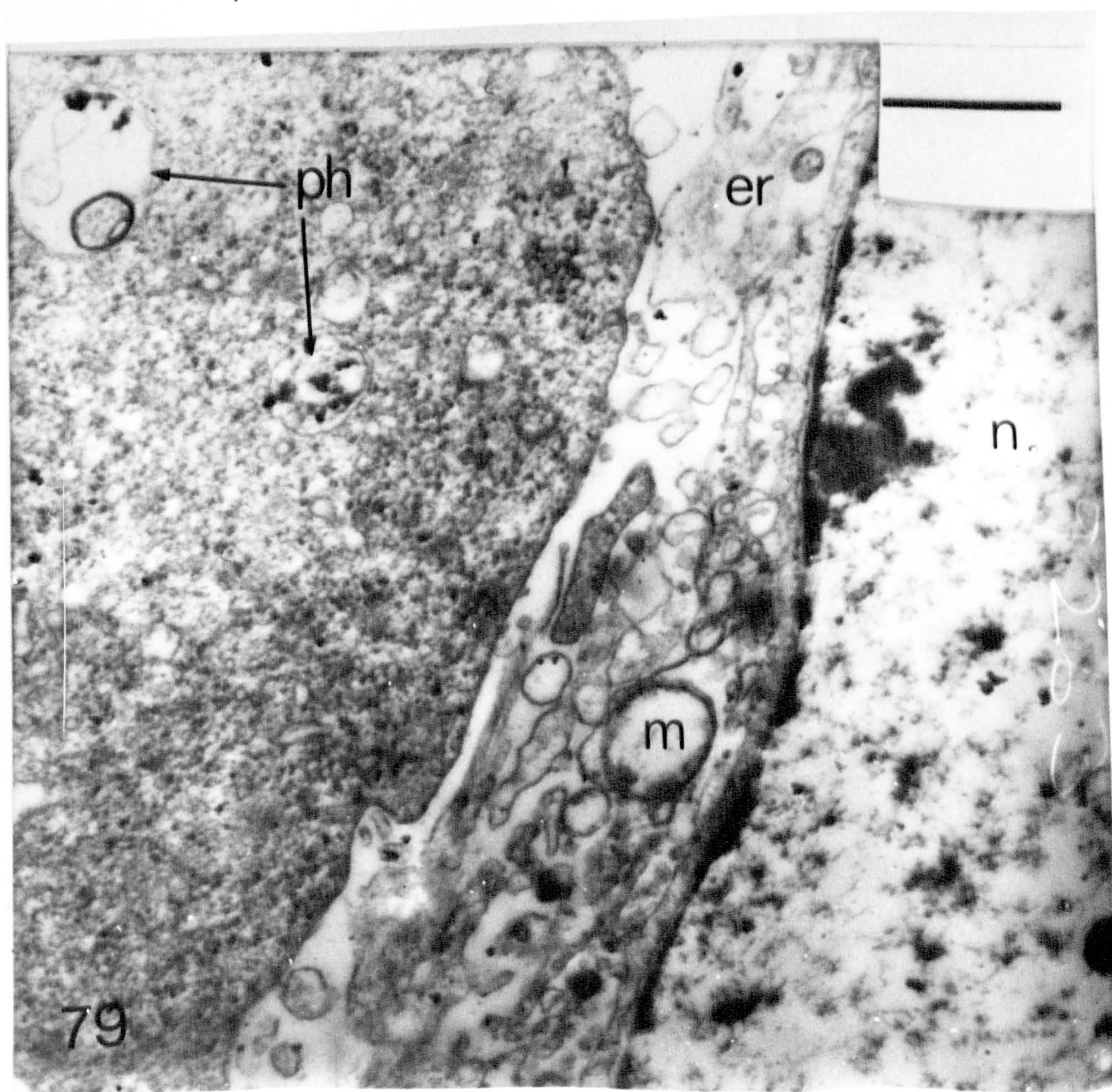
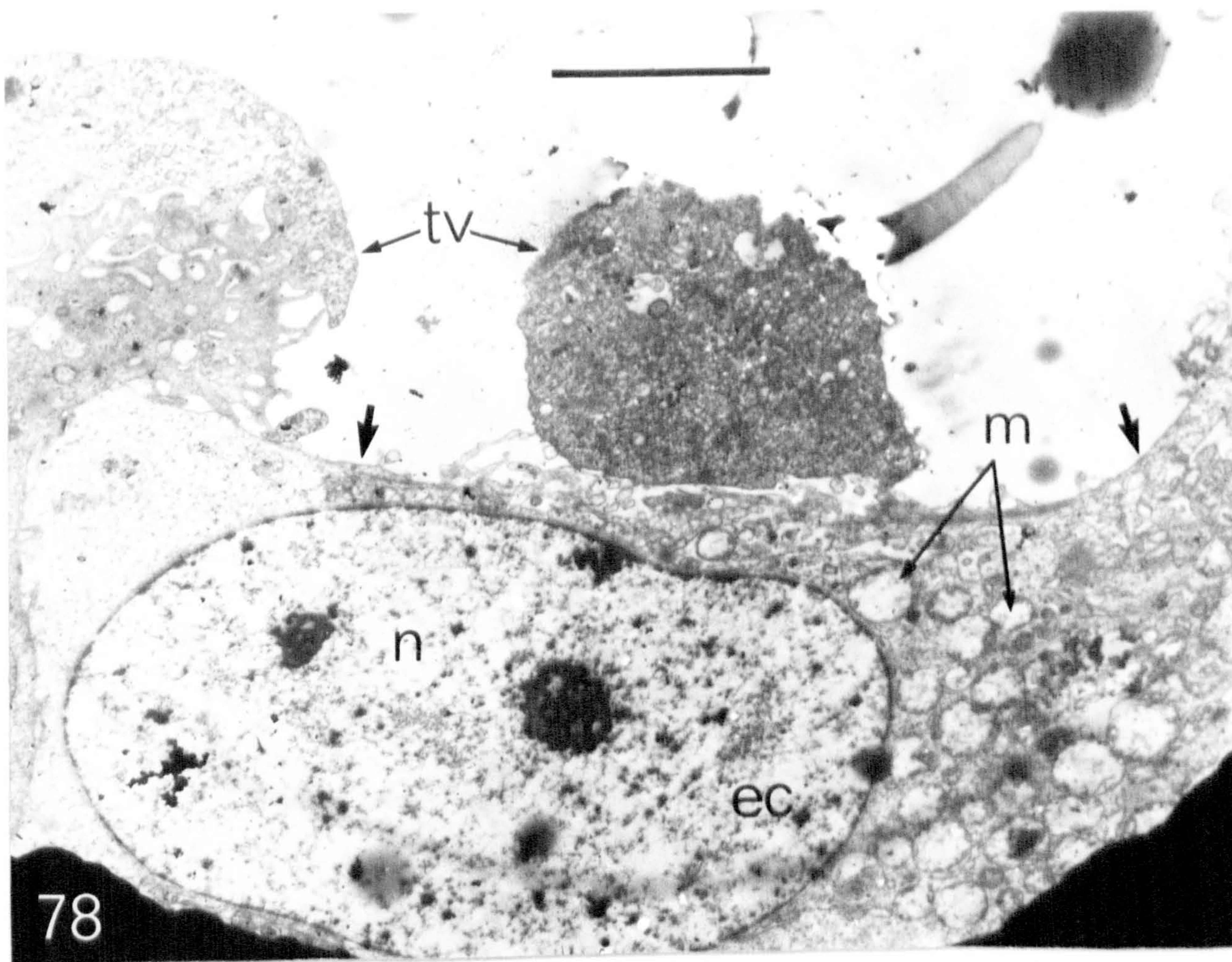
Fig. 78. Low power view showing the morphological changes in the epithelial cell. The plasma membrane of the cell is intact either side of the trichomonad (arrows) but is absent at the points of contact with the trichomonad. The mitochondria (m) are rounded and have lost their cristae. The nucleoplasm and cytoplasm are less electron-dense than normal.

Bar = 4  $\mu$ m

Fig. 79. Detail of Fig. 78 at higher magnification showing the region of contact between T. vaginalis and cell. The plasma membrane of the cell is absent, the endoplasmic reticulum (er) is swollen, ribosomes are absent, mitochondria (m) have lost their cristae and the cytoplasm consists mainly of empty membrane-bound vesicles. Although the nuclear membrane is still complete, the nucleoplasm has lost much of its electron density. Note the phagosomes (ph) within the trichomonad which contain material similar in structure to that in the lysed cell.

Bar = 1  $\mu$ m







Figs. 80 and 81. Phagocytosis of lysed epithelial cells by T. vaginalis.

Fig. 80. Low power view of the edge of a lesion showing T. vaginalis invading a lysed epithelial cell and phagocytosing its contents.

Bar = 5  $\mu$ m

Fig. 81. High magnification detail of Fig. 80 showing the region of phagocytosis. The epithelial cell debris (d) consists of mitochondria, filamentous material (f) and membrane-bound vesicles. The trichomonad has protruded several microfilament-containing pseudopodia (p) which are actively phagocytosing the debris. Some of the debris is already included in phagosomes (ph) within the body of the trichomonad. Note the absence of organelles within the pseudopodia, and the close (20 nm) contact of the pseudopodia with the debris.

Bar = 1  $\mu$ m



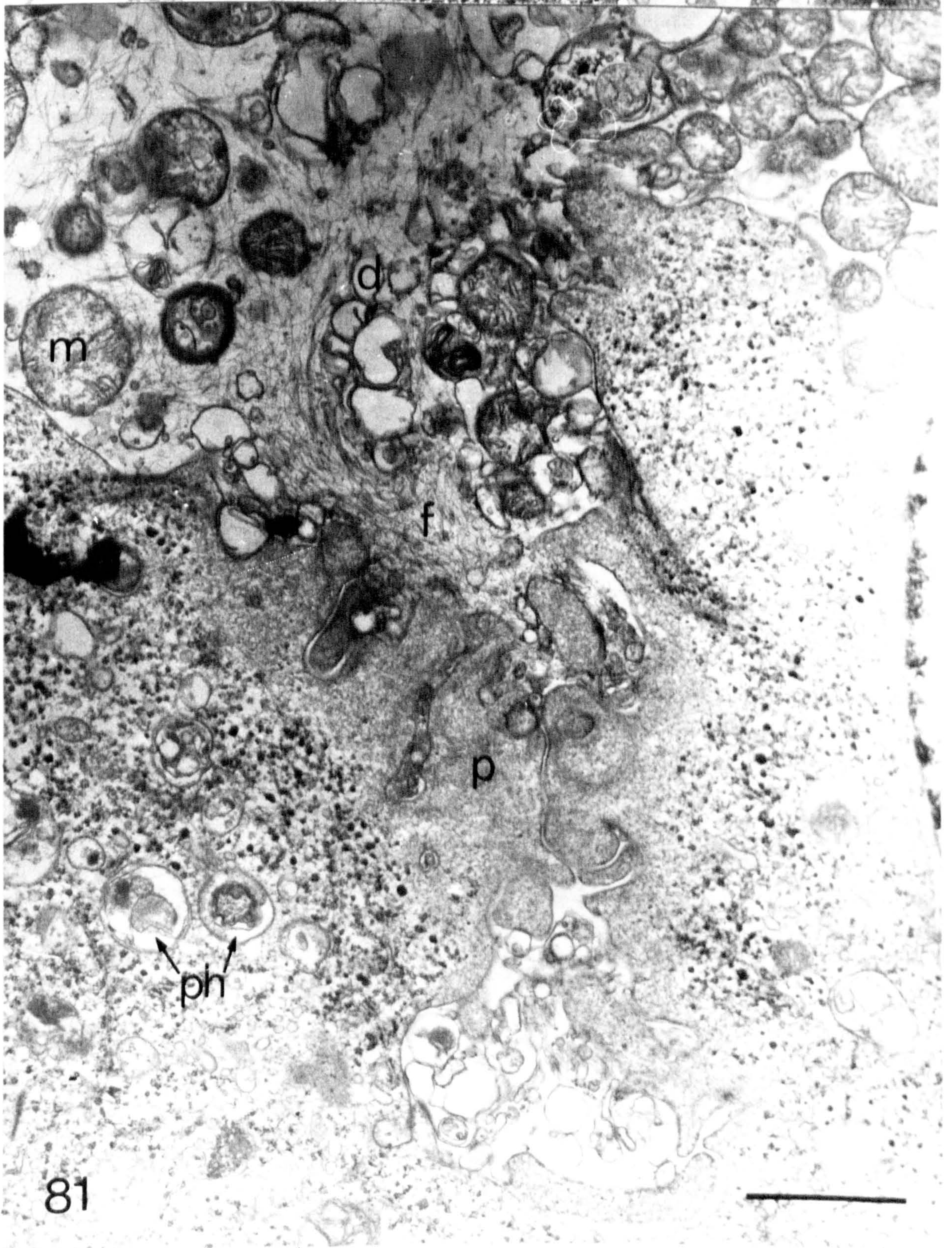
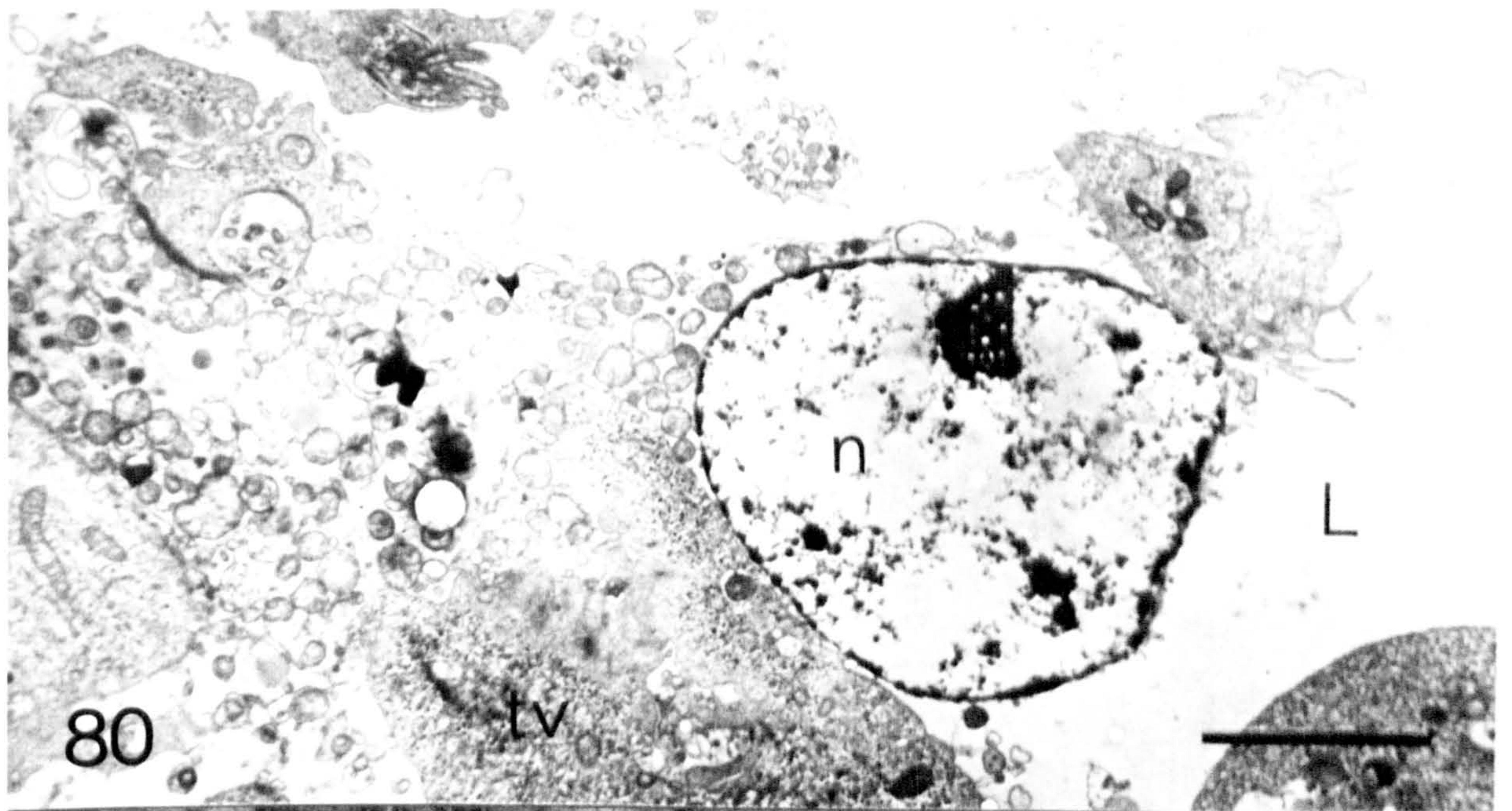




Fig. 82. Phagocytosis of epithelial cell debris by T. vaginalis. High magnification view of an area of T. vaginalis which is engaged in phagocytosis of debris released from a lysed epithelial cell which is shown in Fig. 77. Note the small vesicles (small arrows) 75 nm in diameter which lie in the region of phagocytosis. One vesicle (large arrow) lies next to a large phagosome and may be about to fuse with it.

Bar = 500 nm

Fig. 83. Acid phosphatase-containing vacuoles in the cytoplasm of T. vaginalis. Dense precipitates of lead line the internal surface of the membrane of a large vacuole. A small pinocytotic vesicle also shows enzyme activity (arrow).

Bar = 1  $\mu$ m



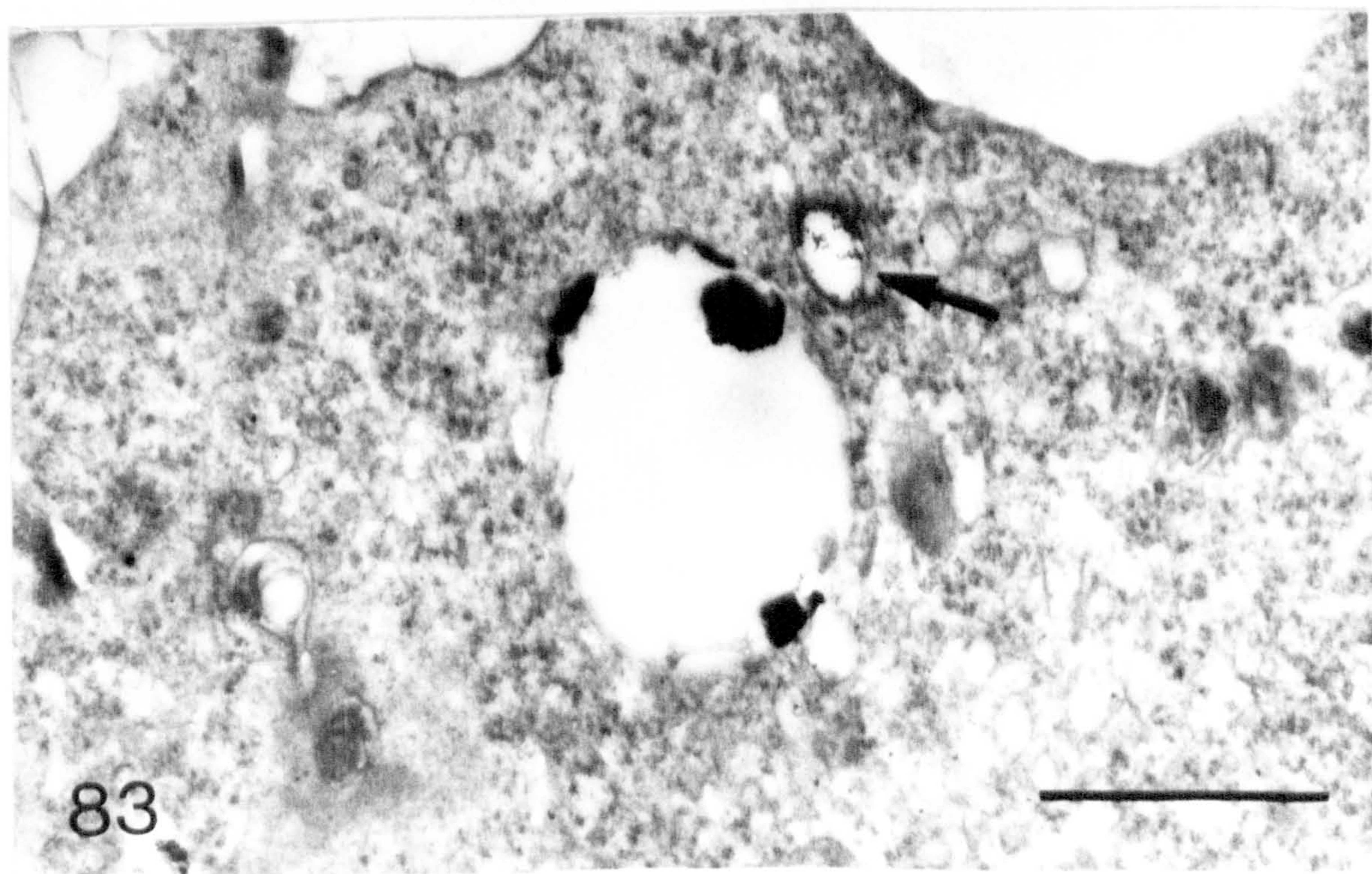
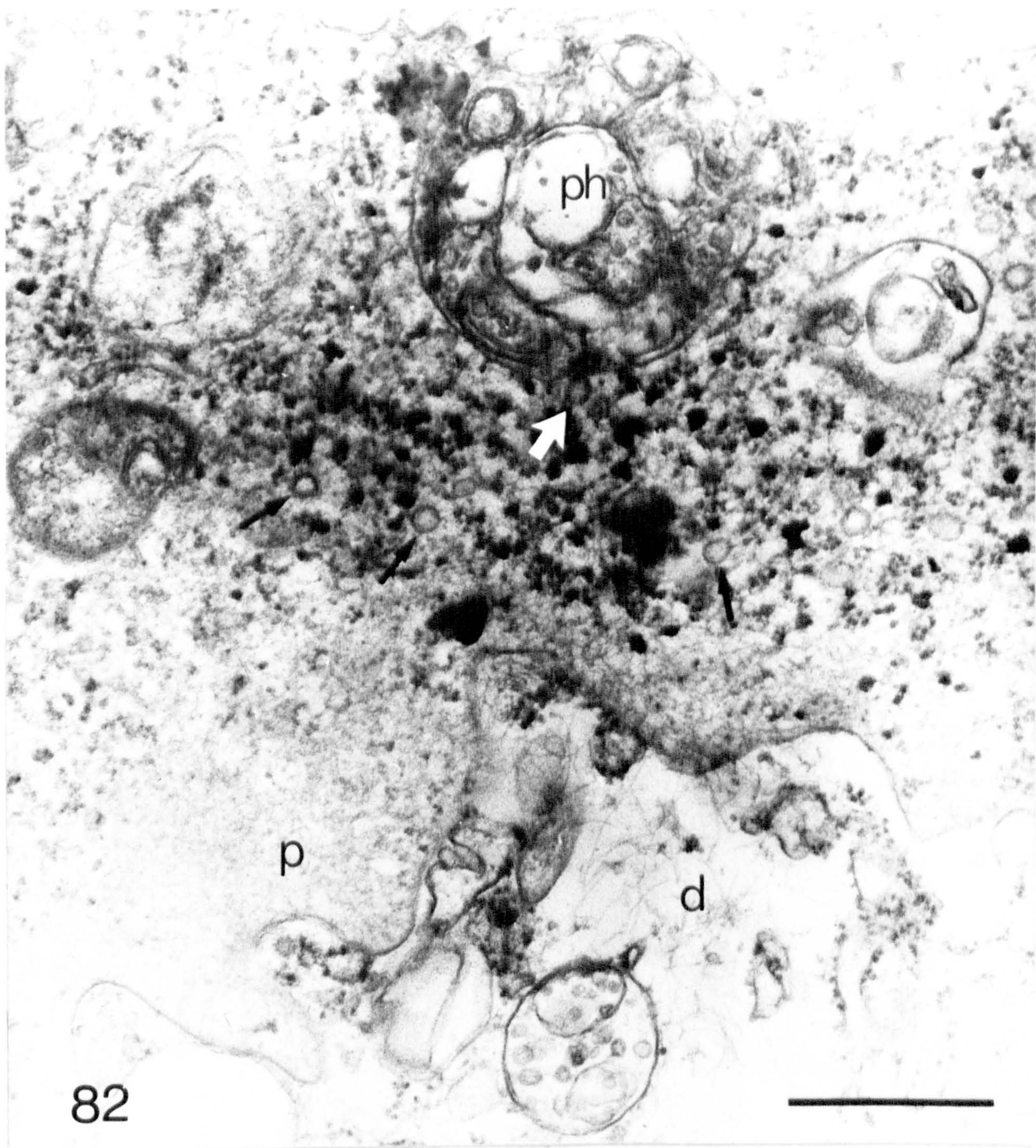




Fig. 84. Phagocytosis of epithelial cell debris by T. vaginalis. Note that the region of phagocytosis is close to the anterior flagella. Two particles of epithelial debris are in the process of being ingested (arrows). Three kinetosomes (k) are seen in this section; that of the recurrent flagellum (rf) lies at an angle of about  $60^\circ$  to those of the other two kinetosomes which lie at the bases of the anterior flagella. Bar = 1  $\mu$ m

Fig. 85. A region of the monolayer lying between the lesions in an RK 13 culture infected for 6 h with T. vaginalis. Note that the epithelial cells shown in this section appear morphologically similar to those cells in uninfected cultures, e.g. Figs. 47 to 49. Bar = 2  $\mu$ m



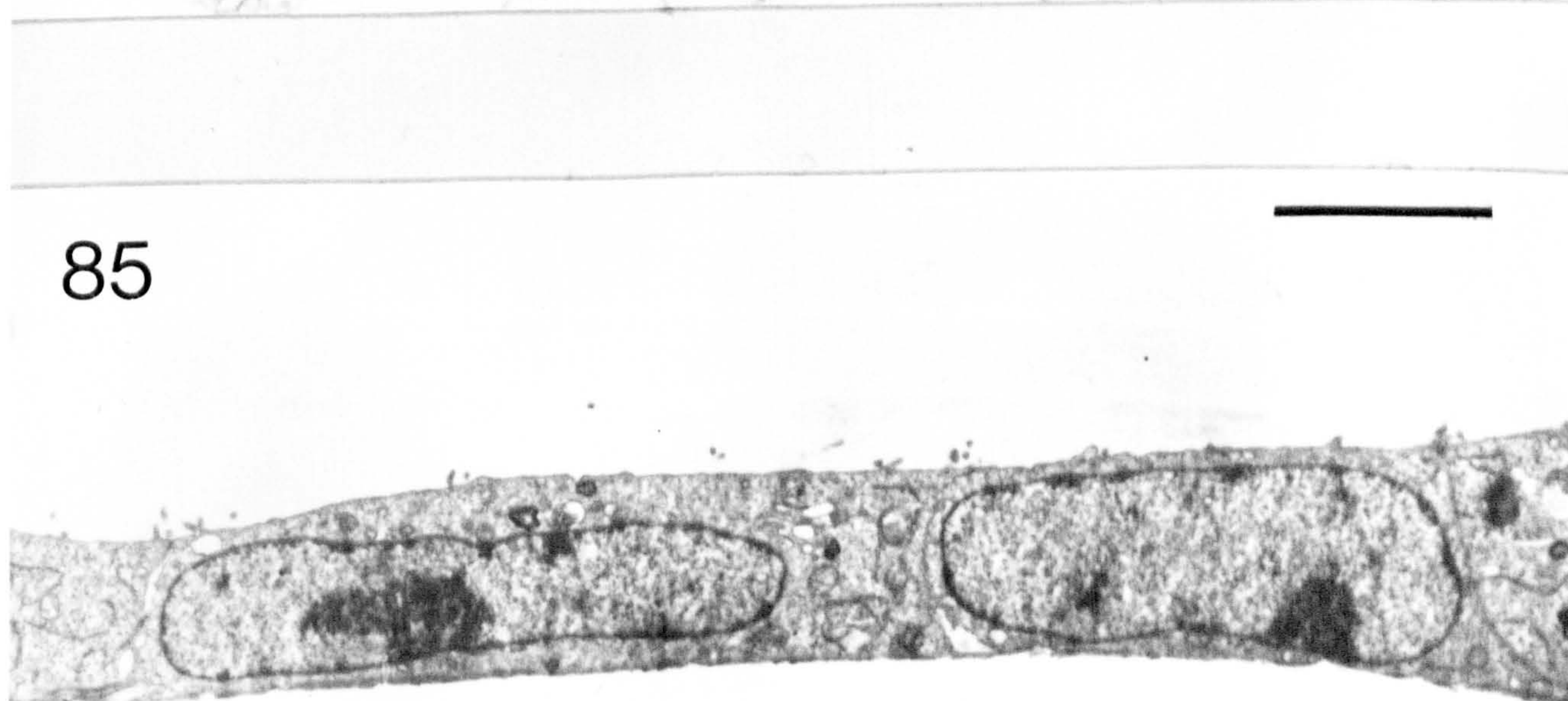
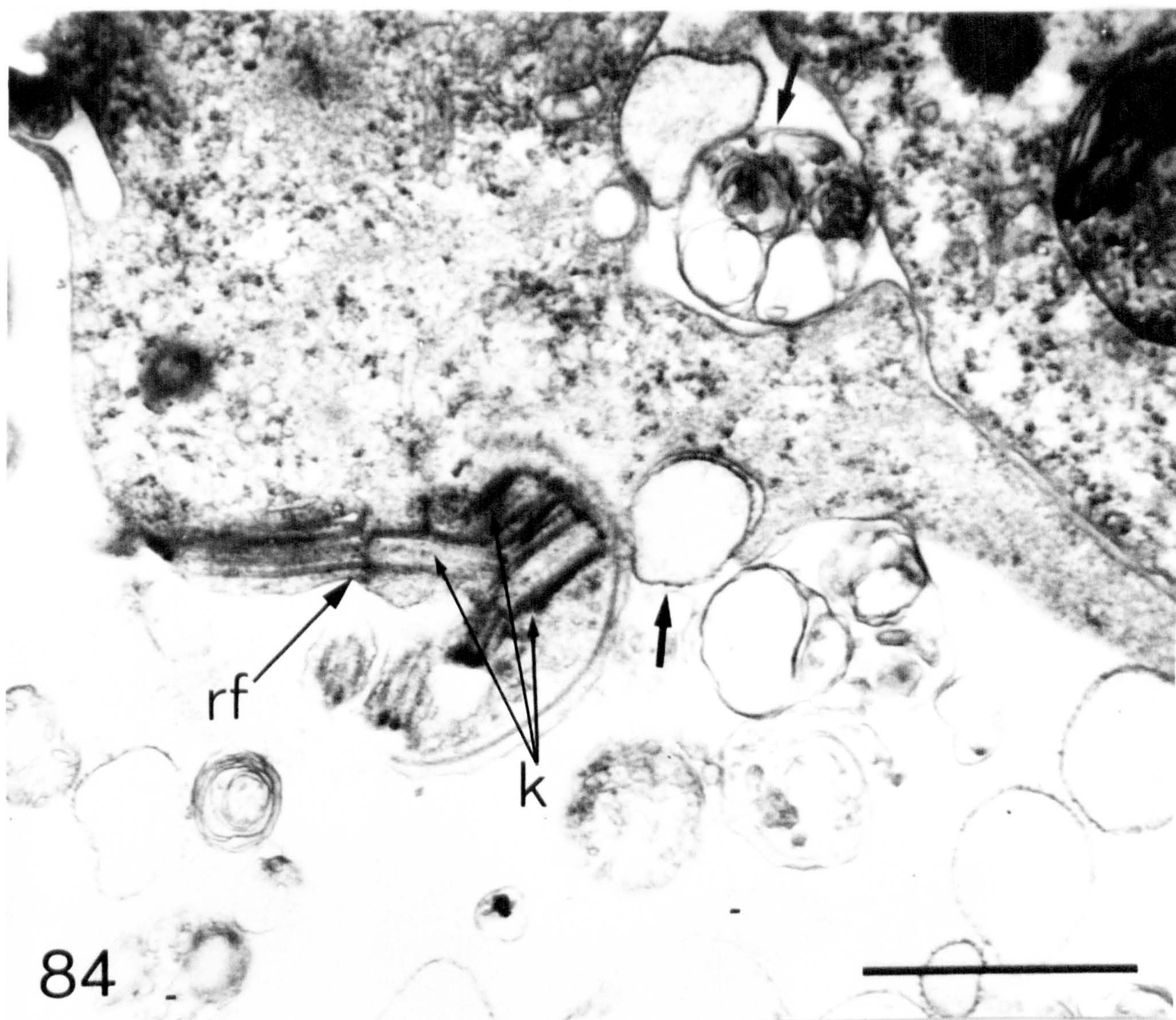




Fig. 86. Phase contrast photograph of a living population of T. vaginalis adhering to a plane glass coverslip in culture. Note the presence of approximately equal numbers of rounded trichomonads (r) (as shown by a peripheral phase-contrast halo) and flattened amoeboid trichomonads (a).

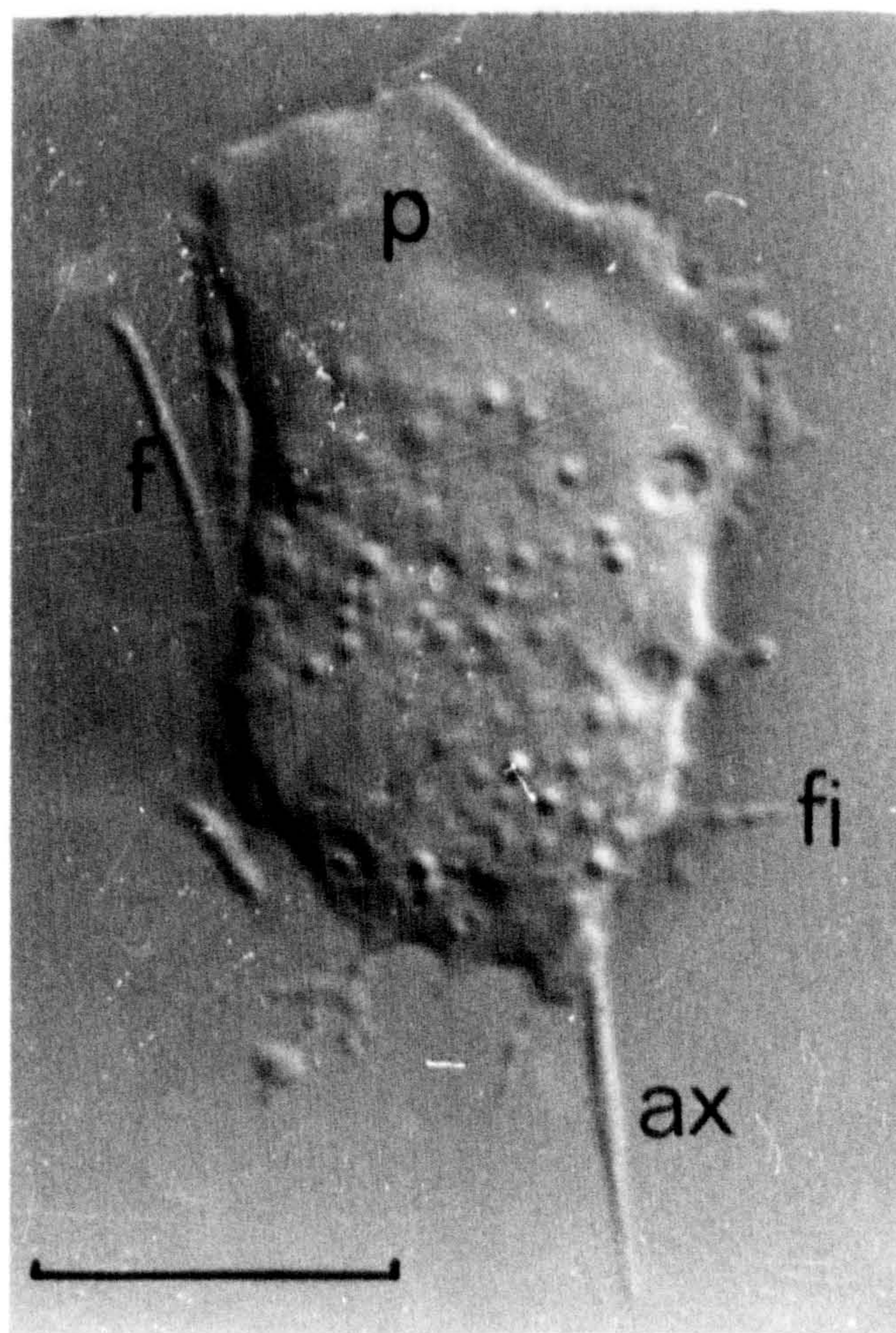
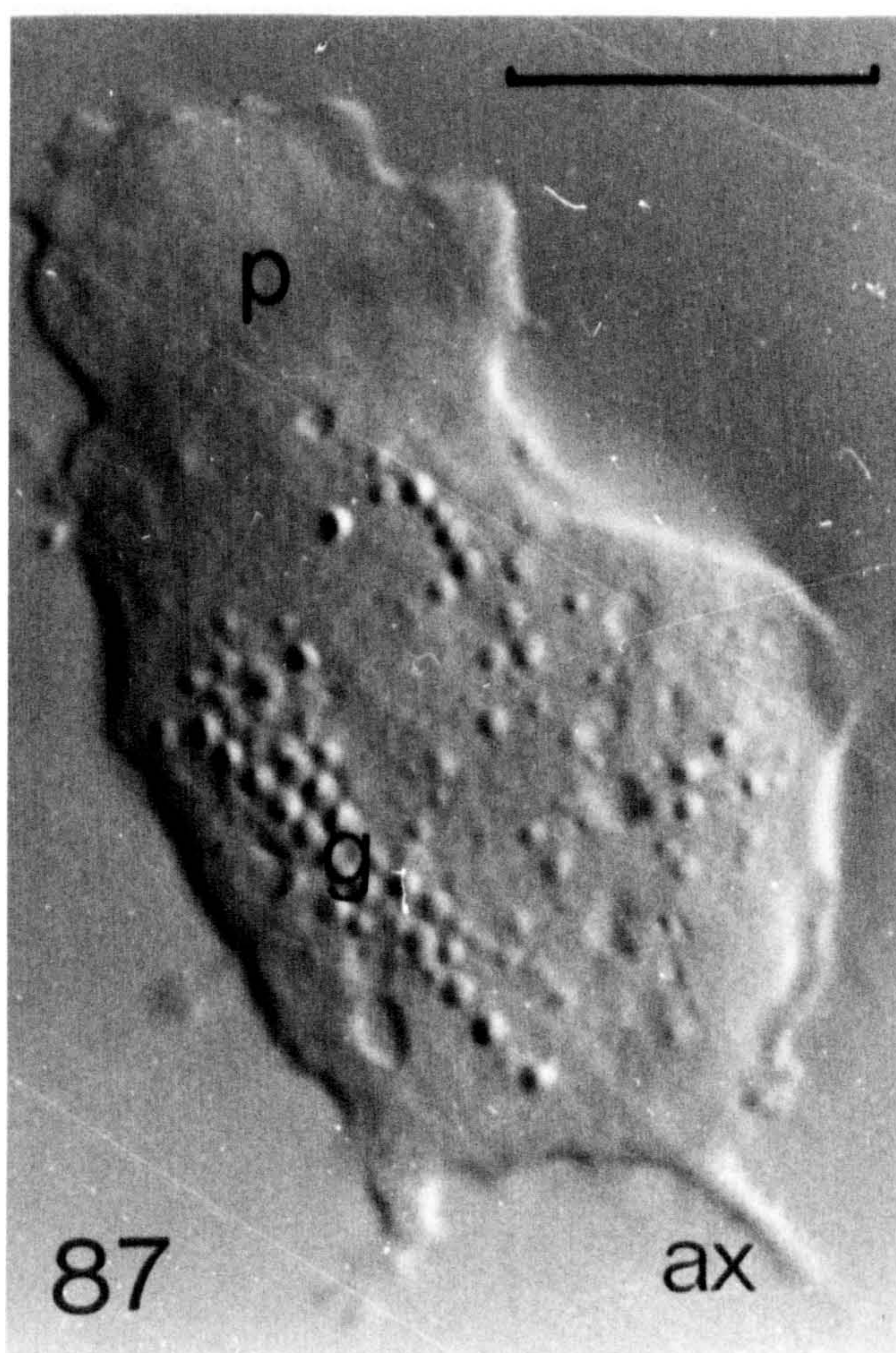
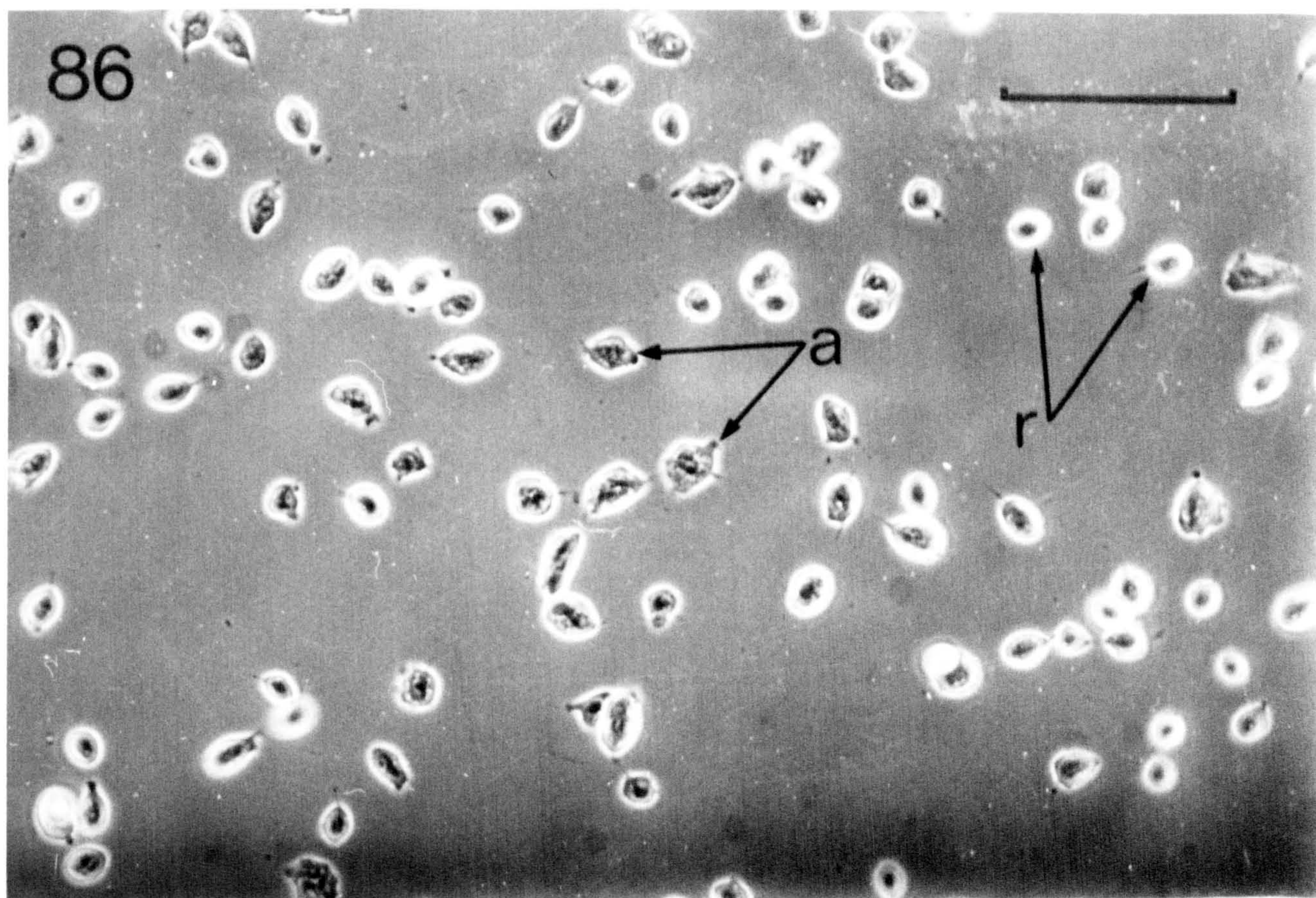
Bar = 100  $\mu$ m

Fig. 87. Nomarski differential interference contrast photographs of living amoeboid T. vaginalis moving on a glass substratum in culture. Note the absence of organelles in the advancing anterior pseudopodia.

p = pseudopodia, ax = axostyle, f = flagella, fi = filopodia, g = cytoplasmic granules (probably hydrogenosomes). The plane of focus is at the substratum-medium interface.

Bar = 10  $\mu$ m







Figs. 88 to 93. Phase contrast photographs of living amoeboid T. vaginalis adhering to a glass substratum showing the diversity in morphology present in a population of organisms.

Bar = 20  $\mu$ m (all photographs are the same magnification)

Fig. 88. A rounded T. vaginalis showing the axostyle (ax), undulating membrane (um) and an irregularly shaped lateral pseudopodium (p). Note the filopodial processes around the periphery.

Fig. 89. A trichomonad which is polarised along its antero-posterior axis. The anterior flagella (af) and undulating membrane (um) can be seen at the anterior pole. A single pseudopodium (p) extends from the posterior pole. Note the absence of cytoplasmic granules in the pseudopodium.

Fig. 90. A flattened T. vaginalis with filopodial projections (fi).

Fig. 91. Cytokinesis in a dividing T. vaginalis. The two daughter trichomonads are joined by a cytoplasmic bridge (arrow) and are about to separate.

Fig. 92. A large flattened amoeboid T. vaginalis. A phagosome (ph) can be seen within the cytoplasm.

Fig. 93. A dividing T. vaginalis. Two sets of flagella (f) are present.



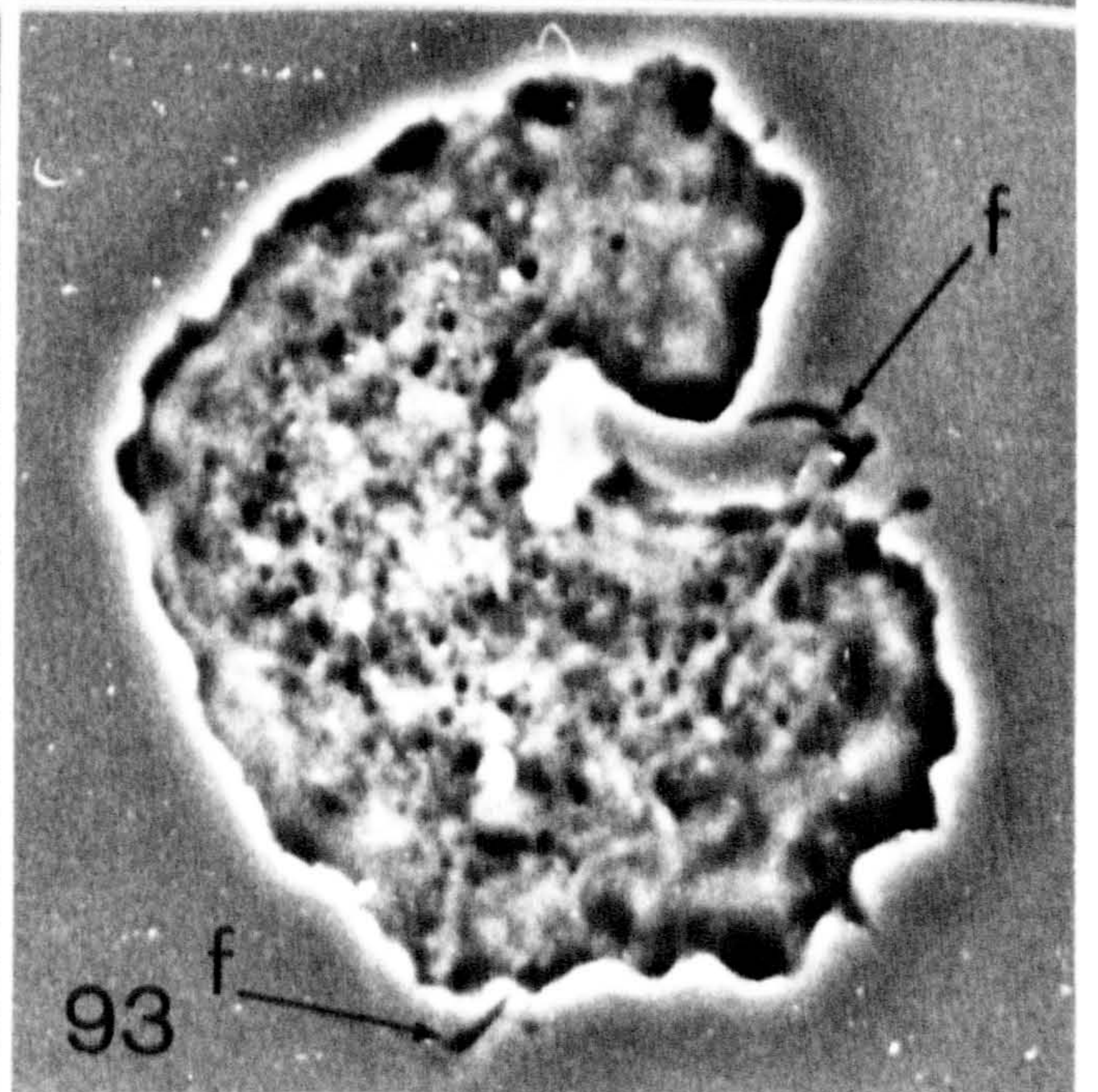
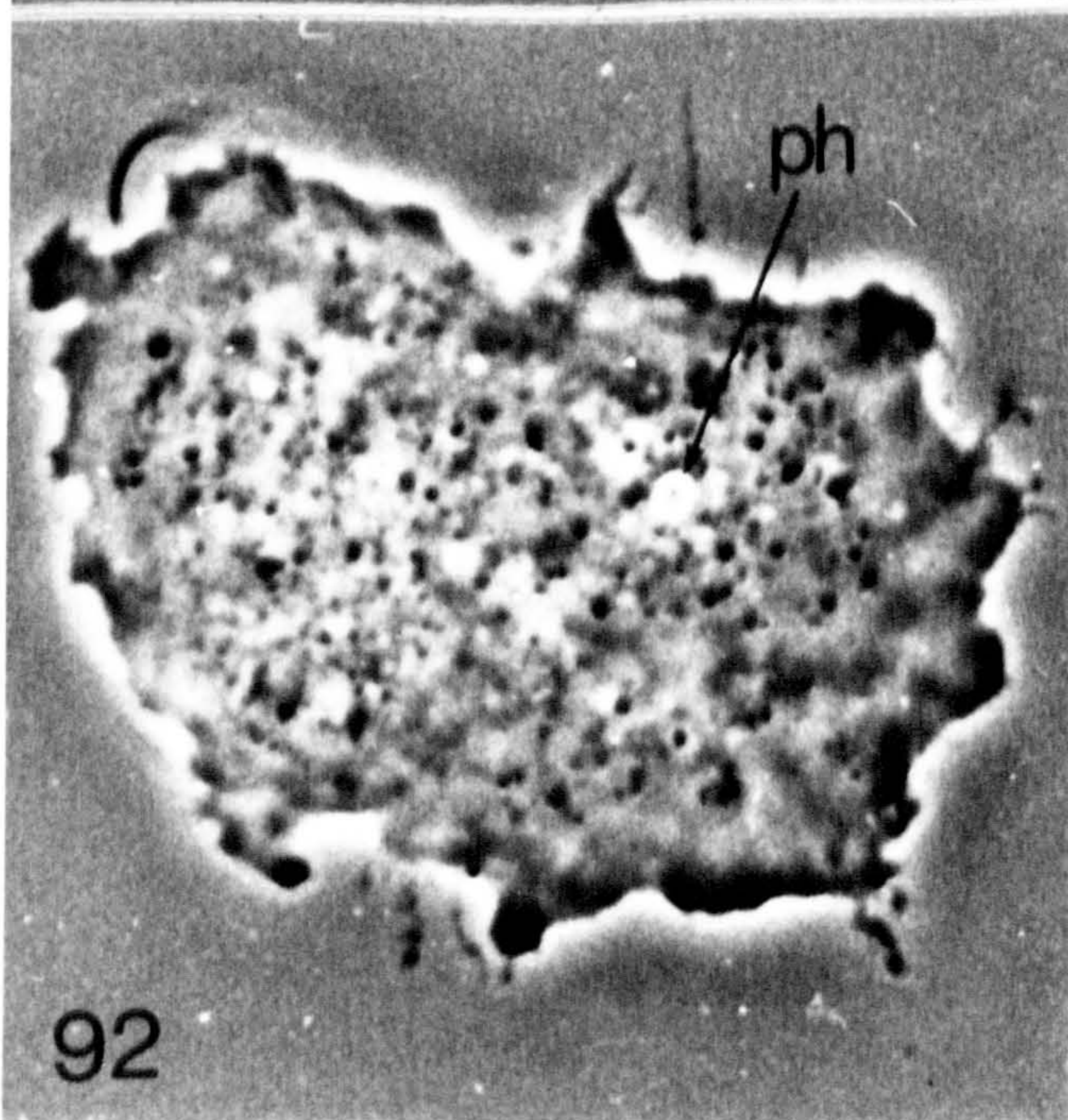
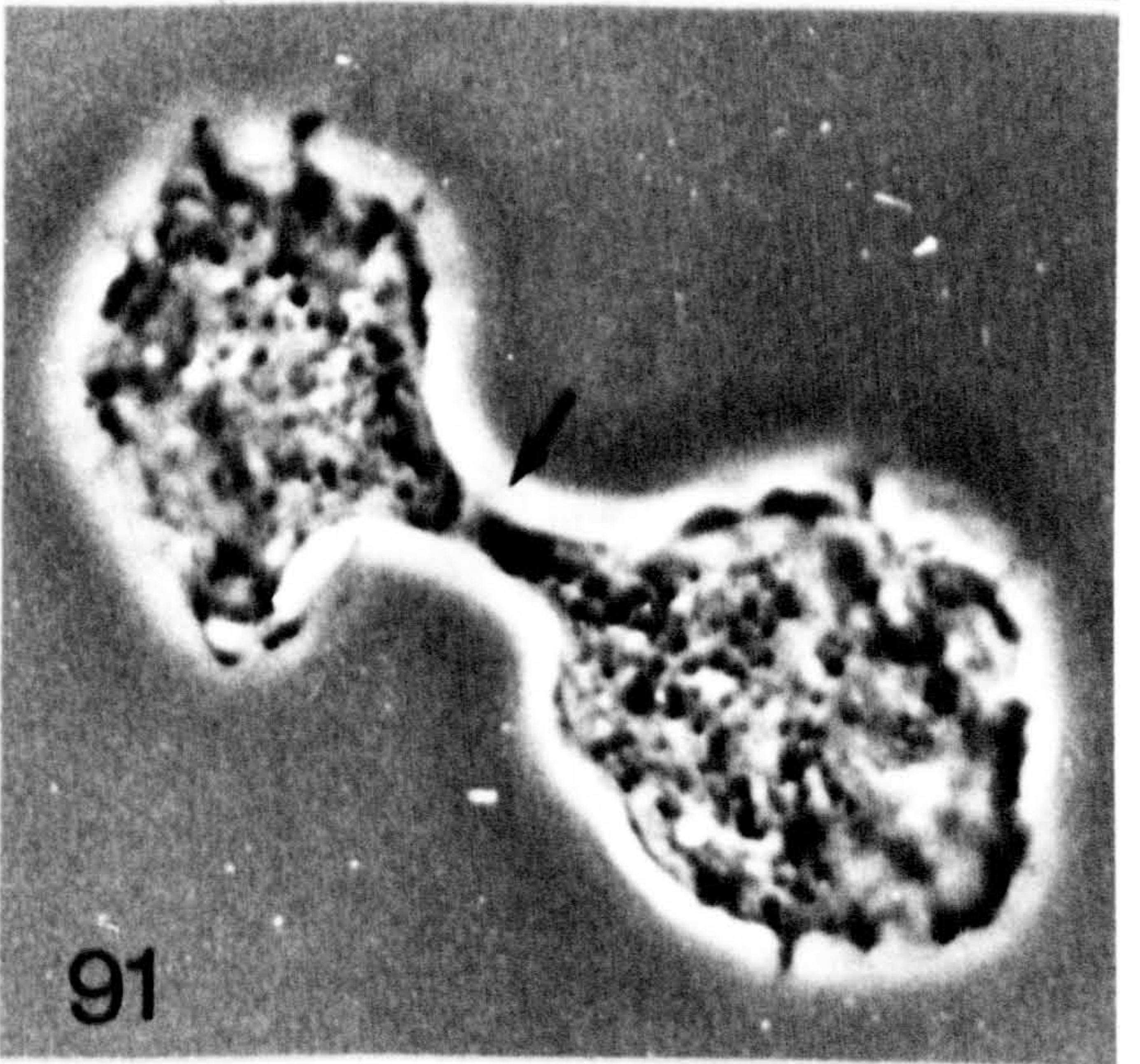
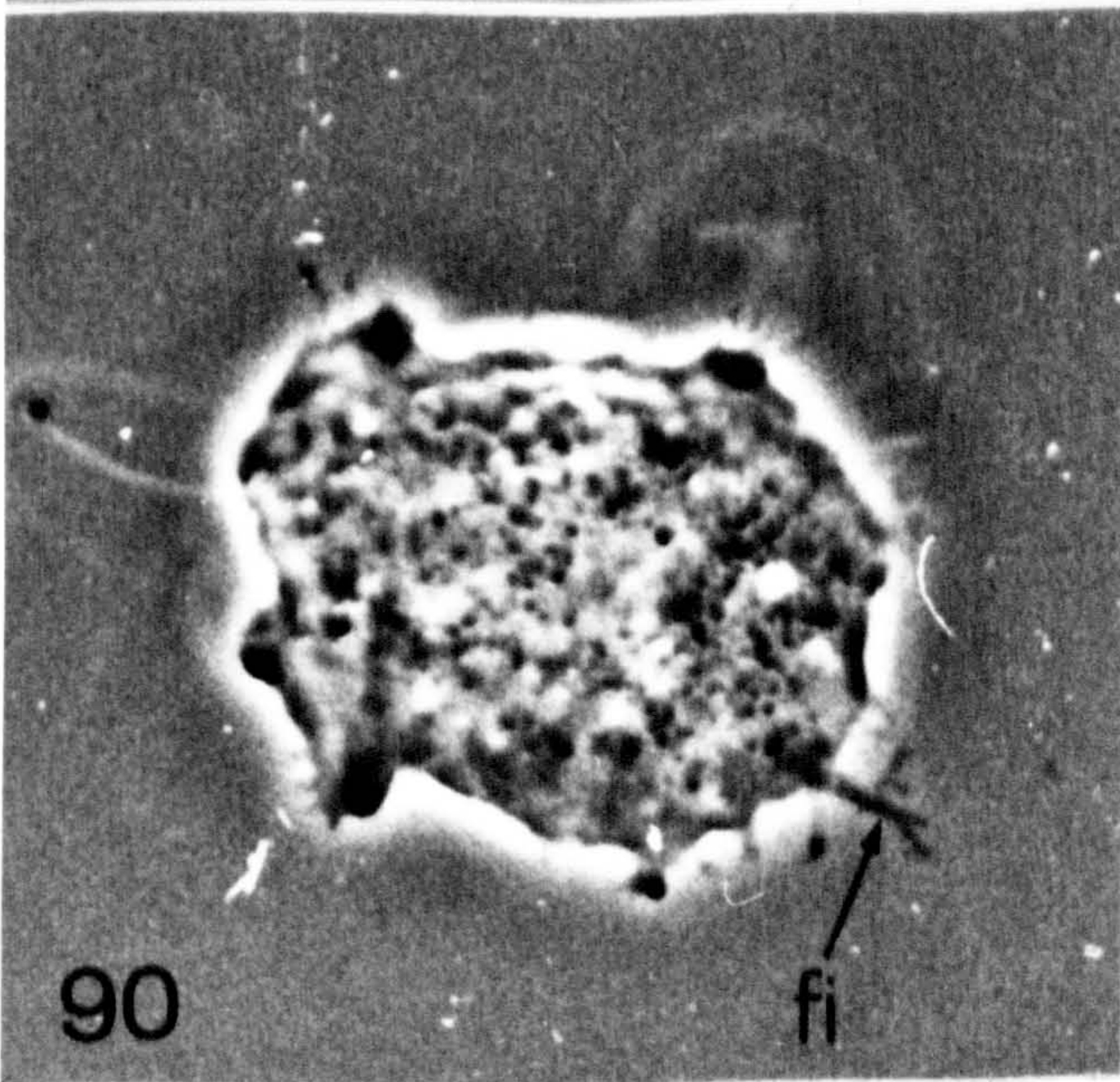
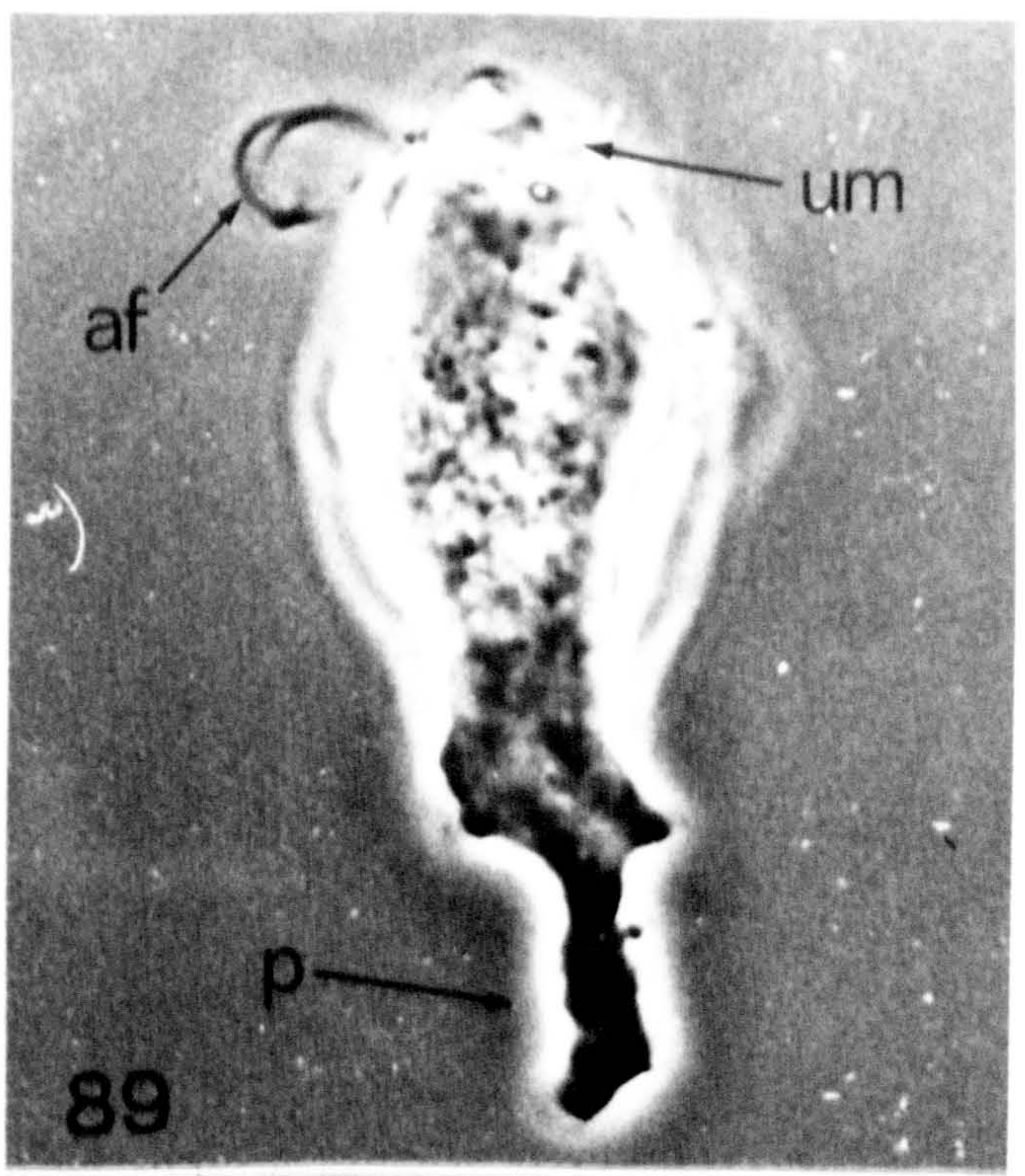
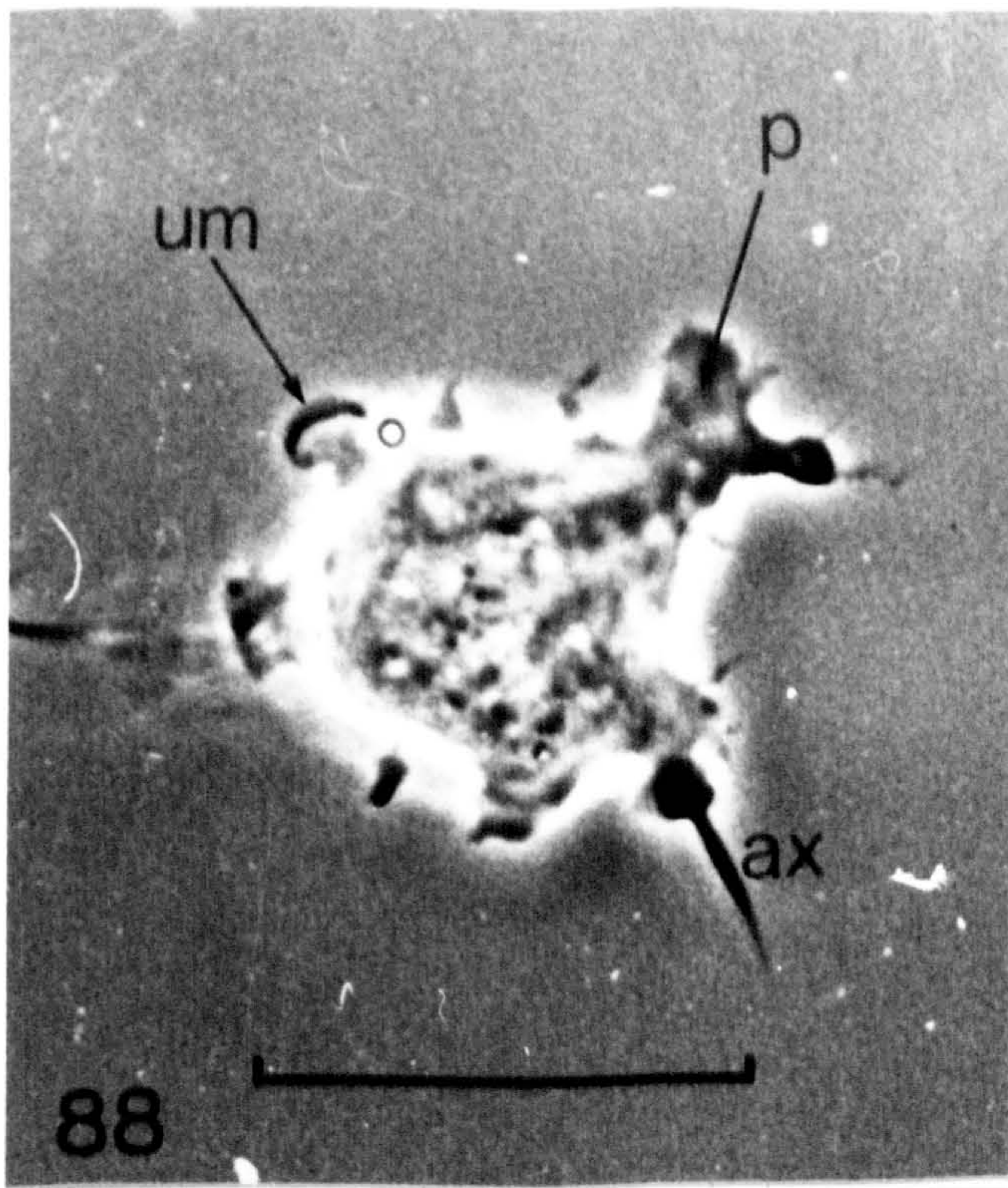




Fig. 94. A time-lapse sequence at 10 second intervals of Nomarski differential interference contrast photographs of a living amoeboid T. vaginalis showing the motility of the organism on a glass substratum in culture. Each frame shows the same area of the substratum. In the 50 second period the organism changes from an irregular to a more polarised form and moves to the right of the field by extending a pseudopodium (arrow). The mean speed of the organism over 50 seconds is about 20  $\mu\text{m}$  per min.

Bar = 30  $\mu\text{m}$



94

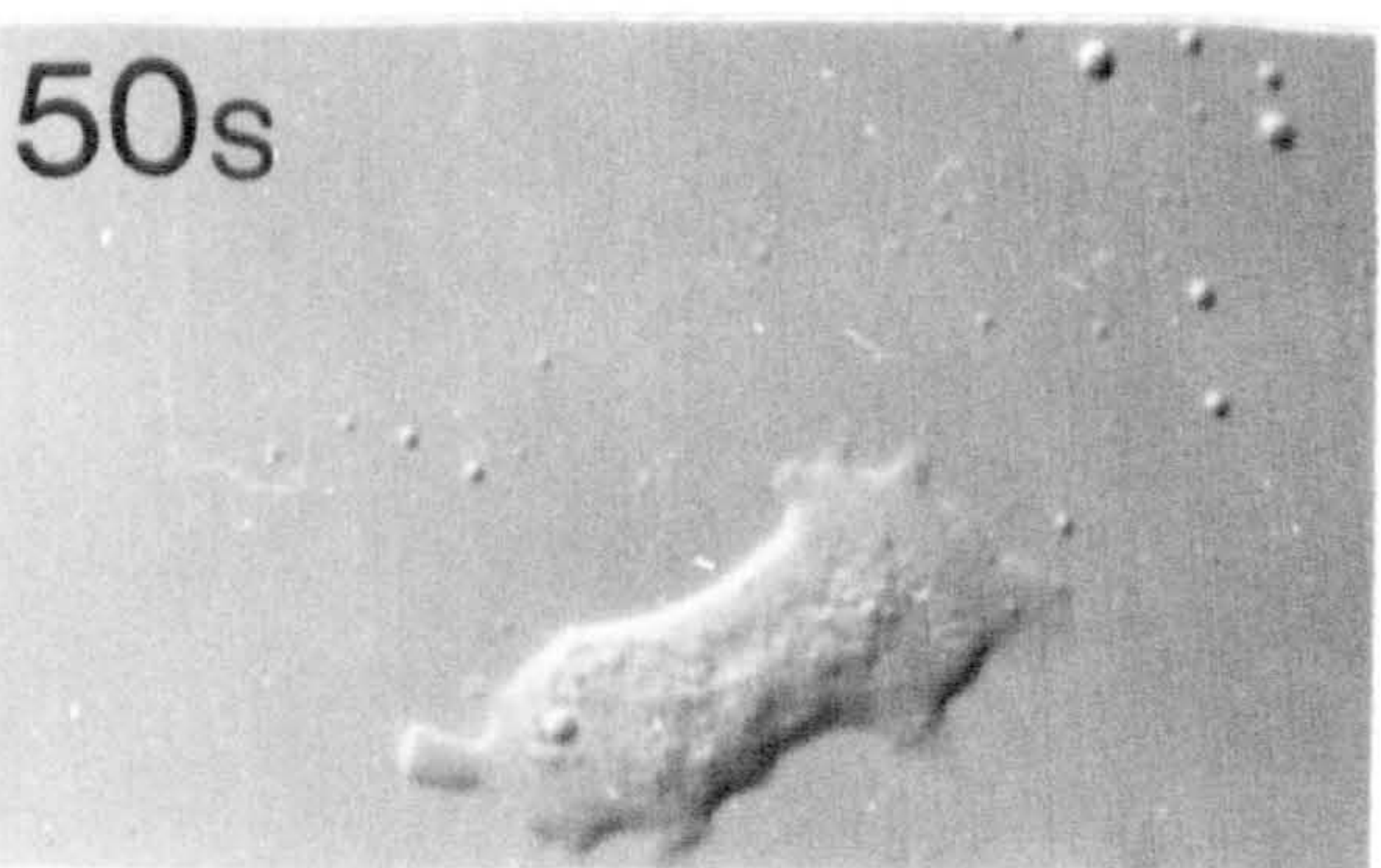
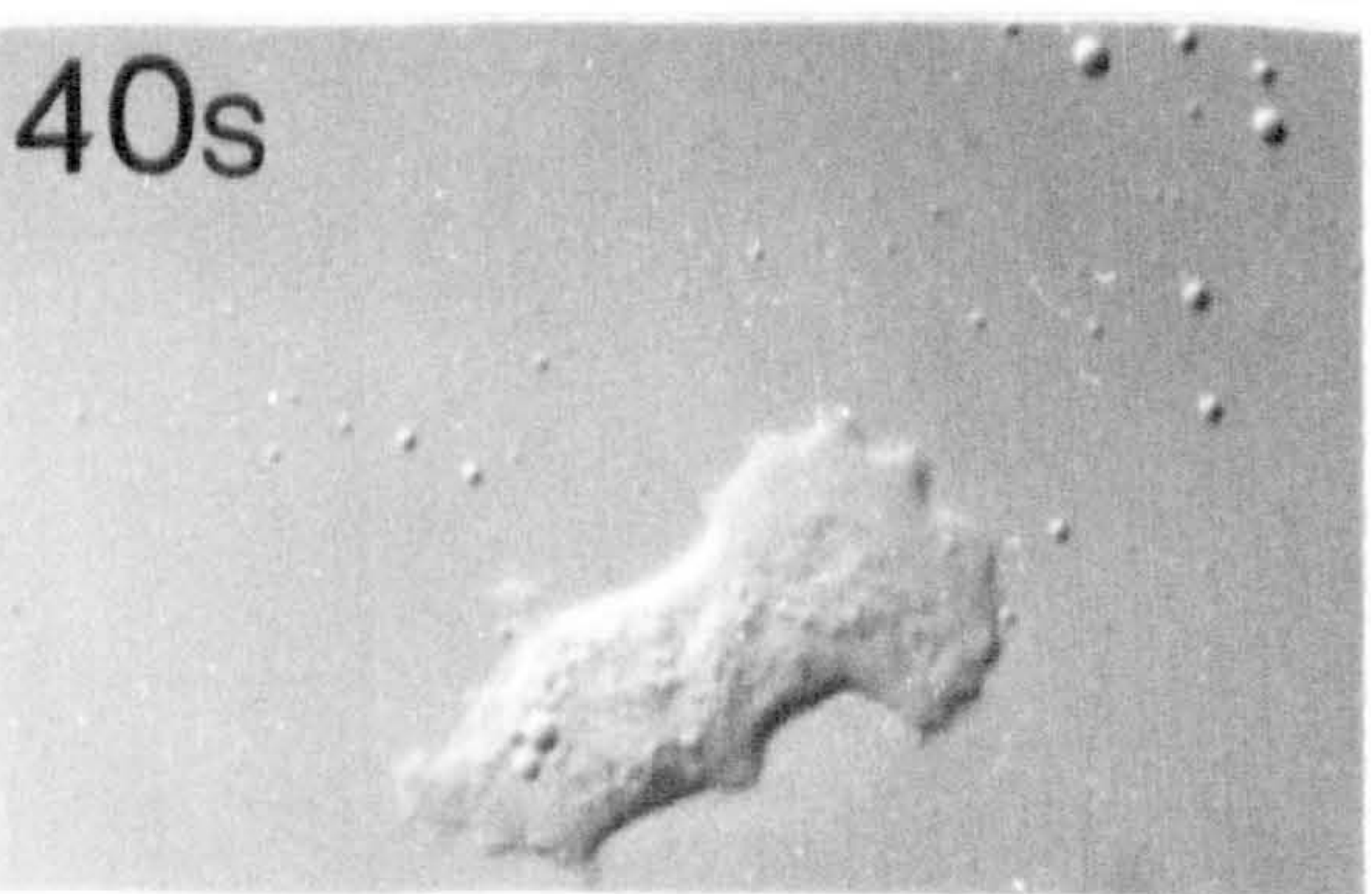
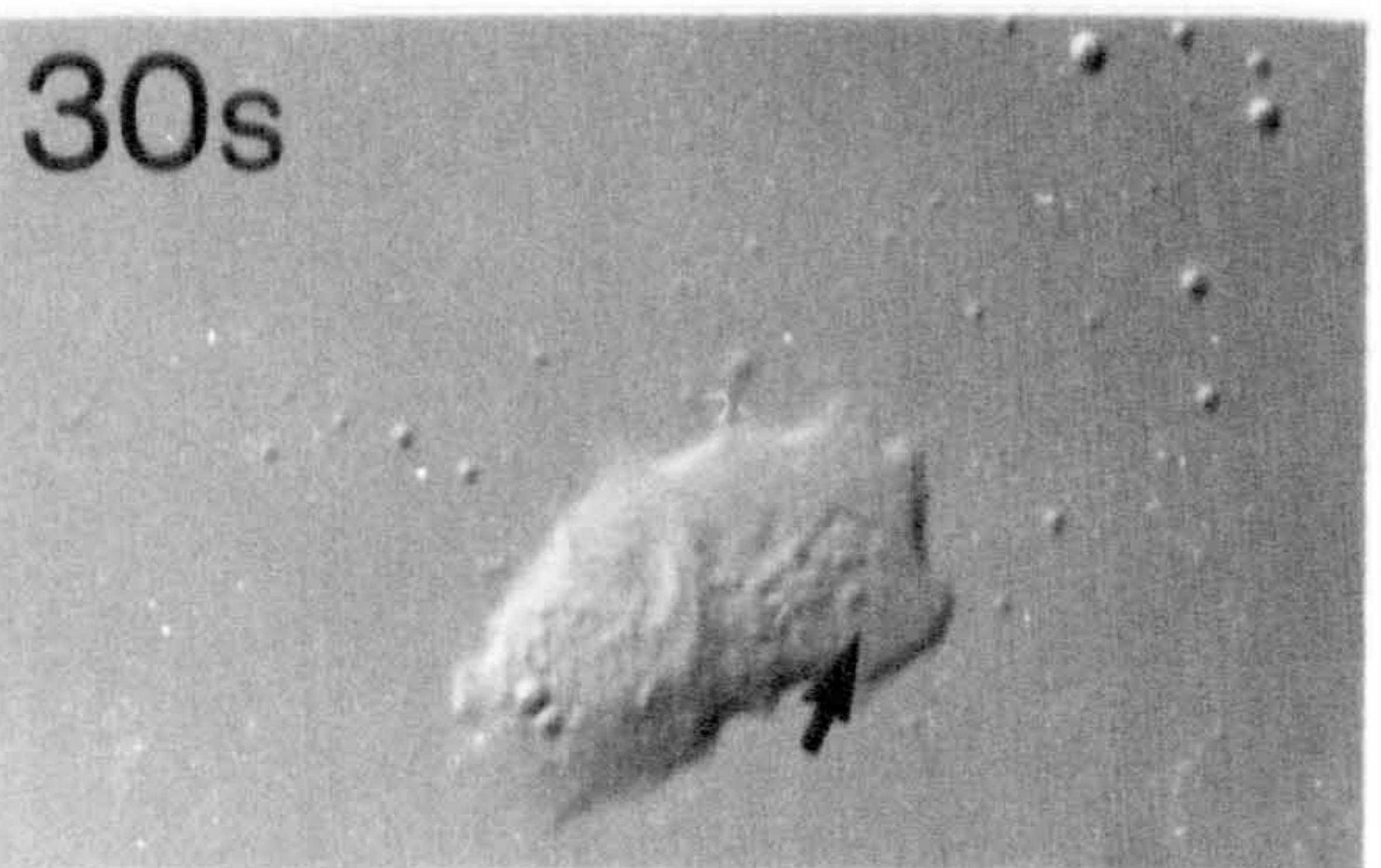
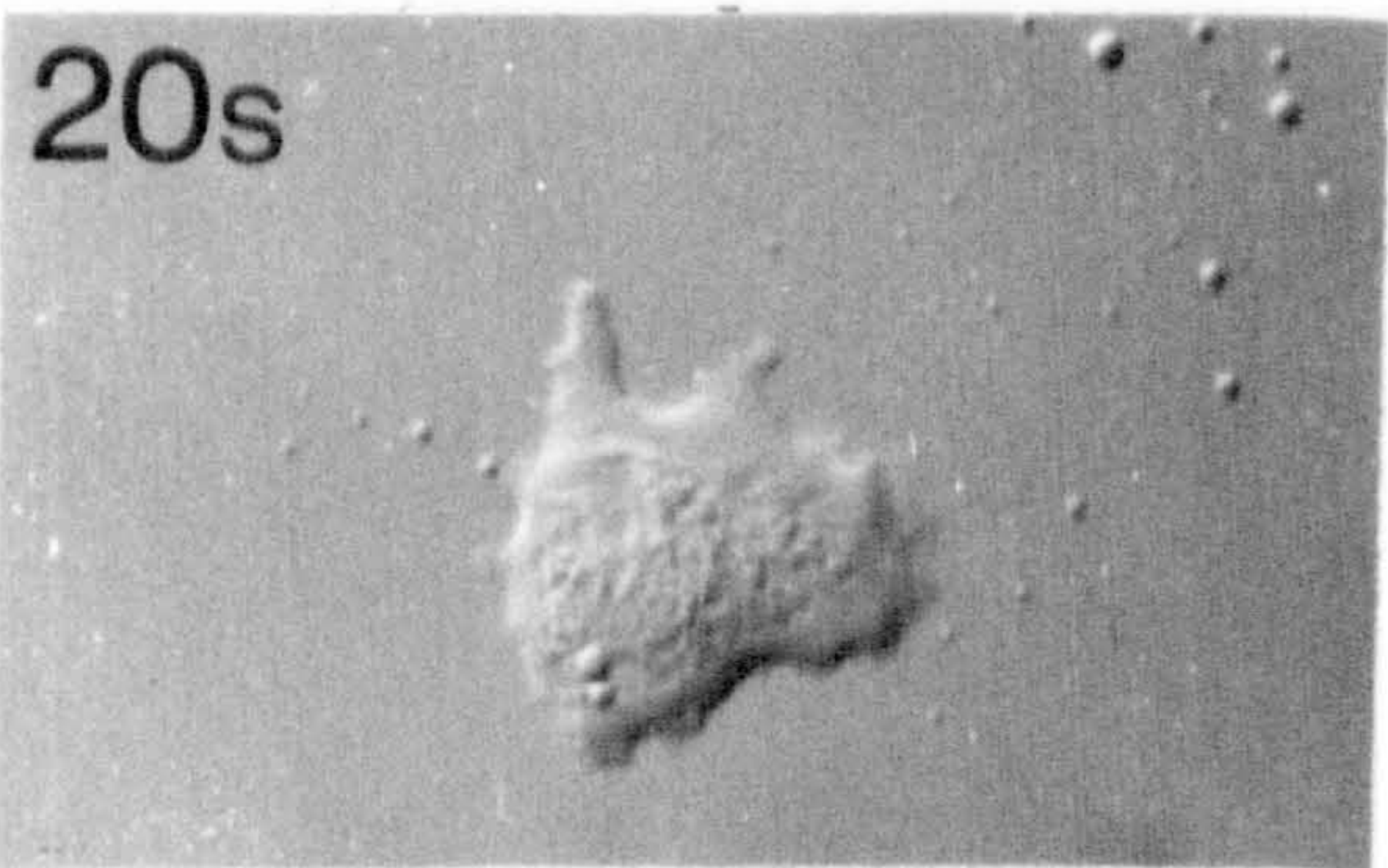
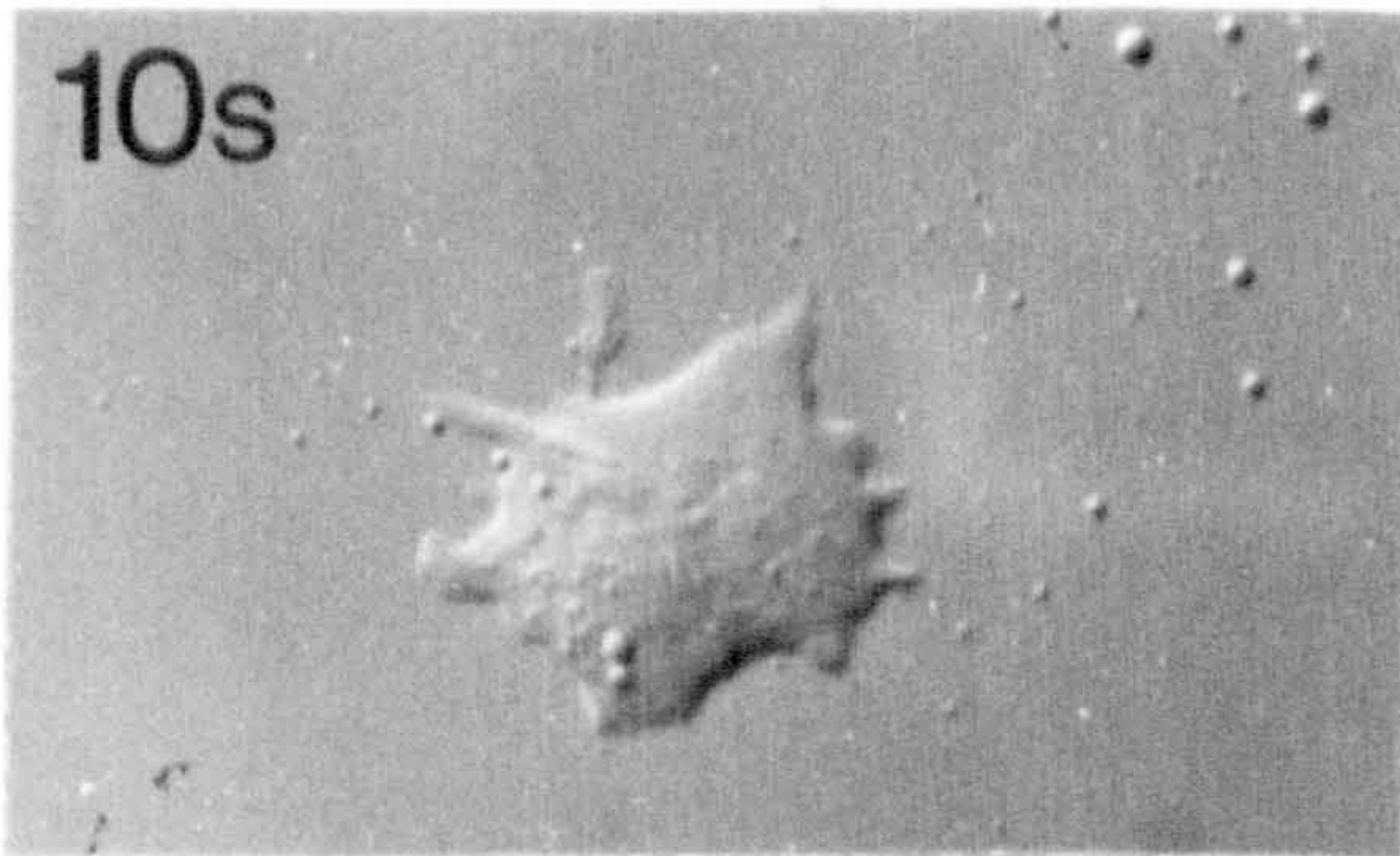
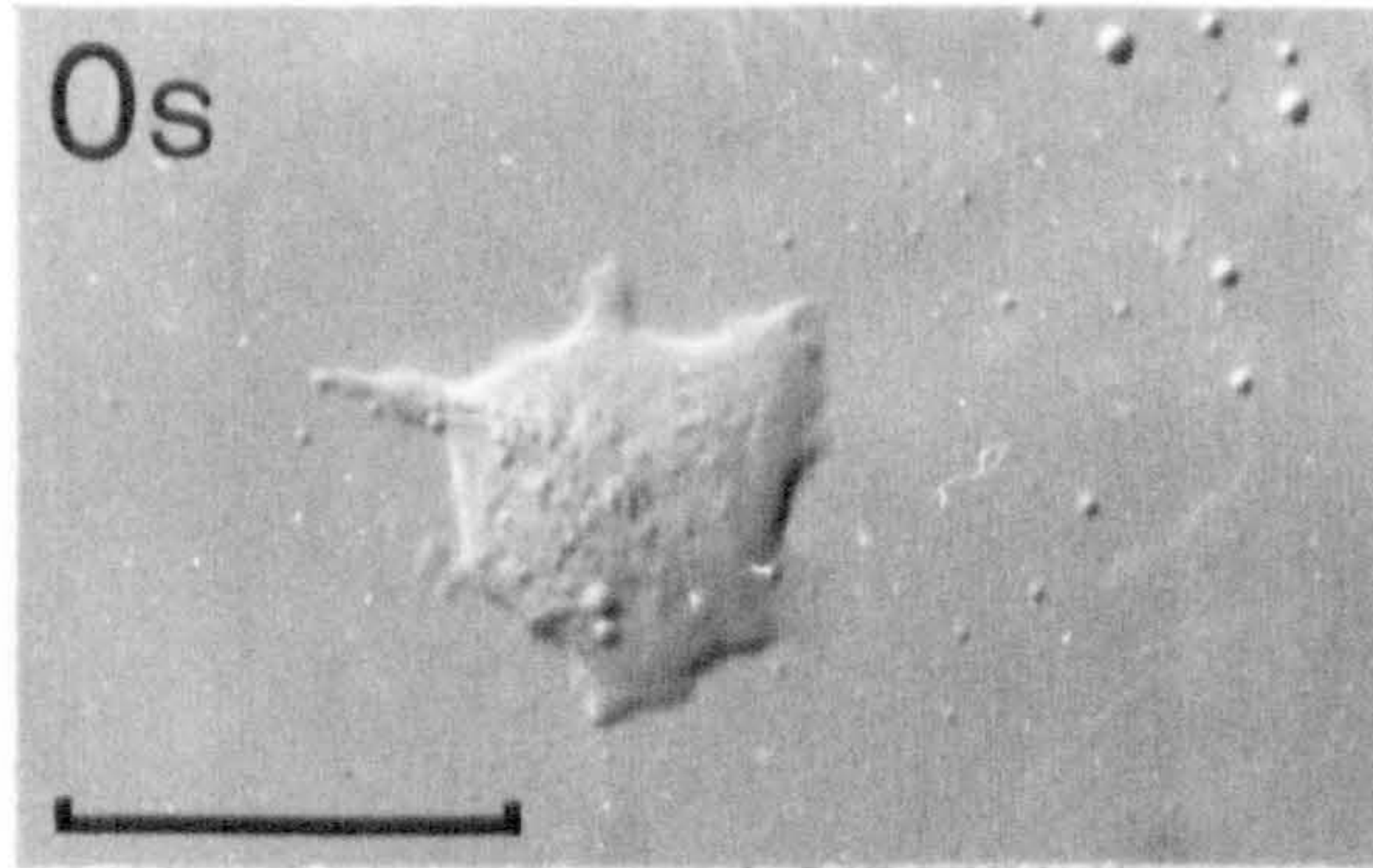




Fig. 95. A time-lapse sequence of phase contrast photographs of a living amoeboid T. vaginalis showing the motility of the organism on a glass substratum in culture. Each frame, taken at 1 min. intervals, shows the same area of the substratum. Over the 11 min. period the path of the trichomonad describes a circle; the organism continuously protrudes pseudopodia from its periphery but generally moves in the direction of protrusion of its largest pseudopodium e.g. frames 2 min. to 5 min.

Bar = 30  $\mu$ m



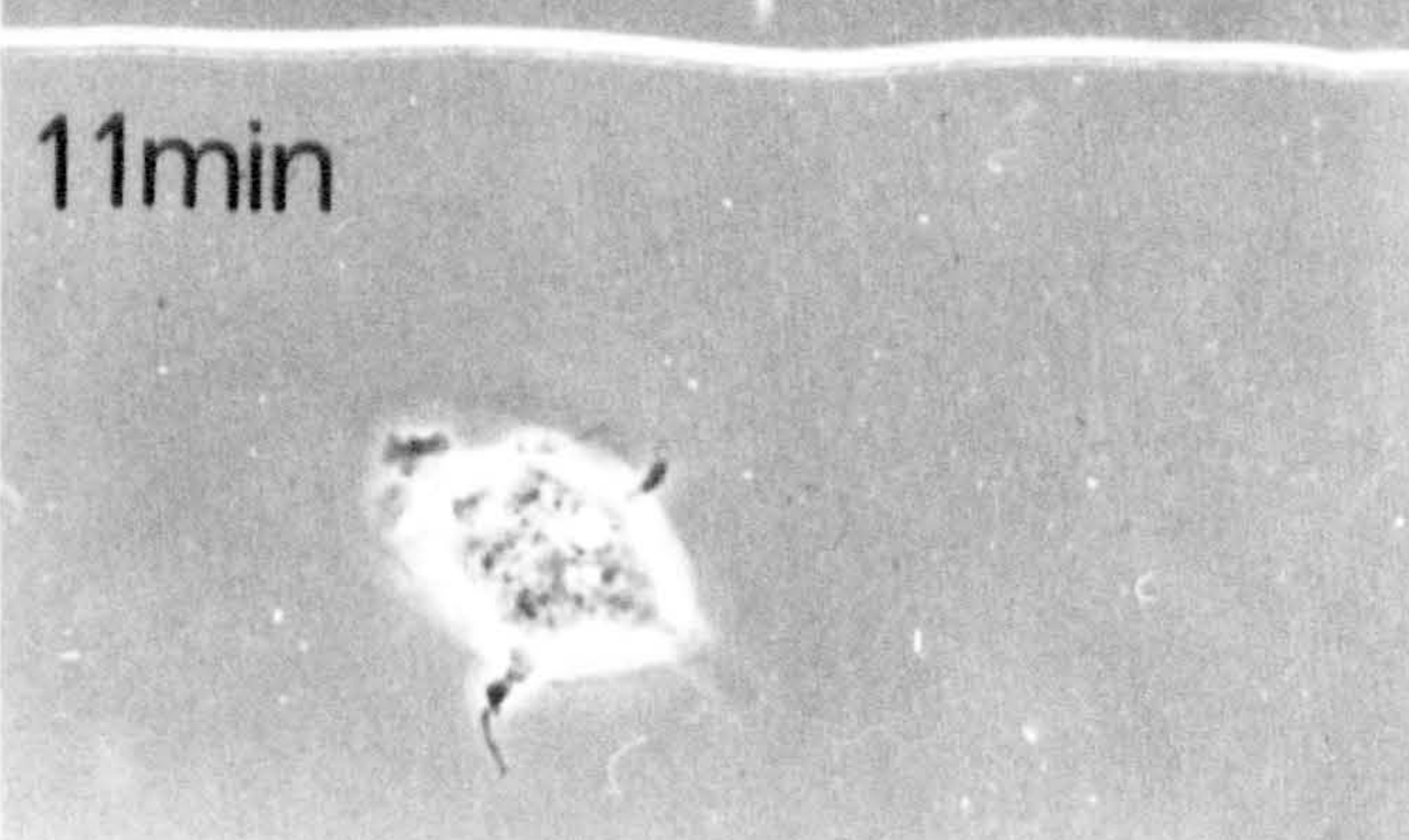
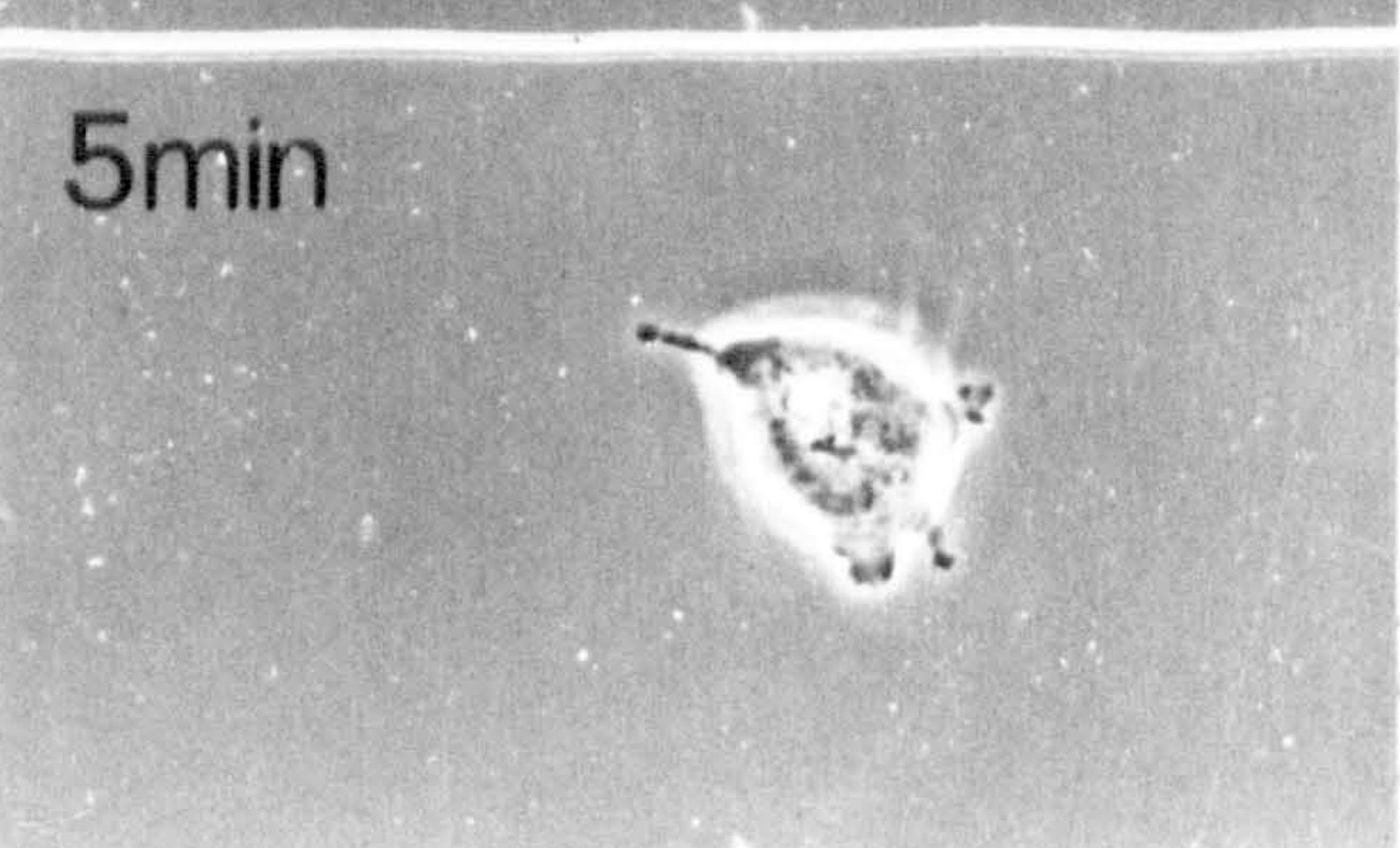
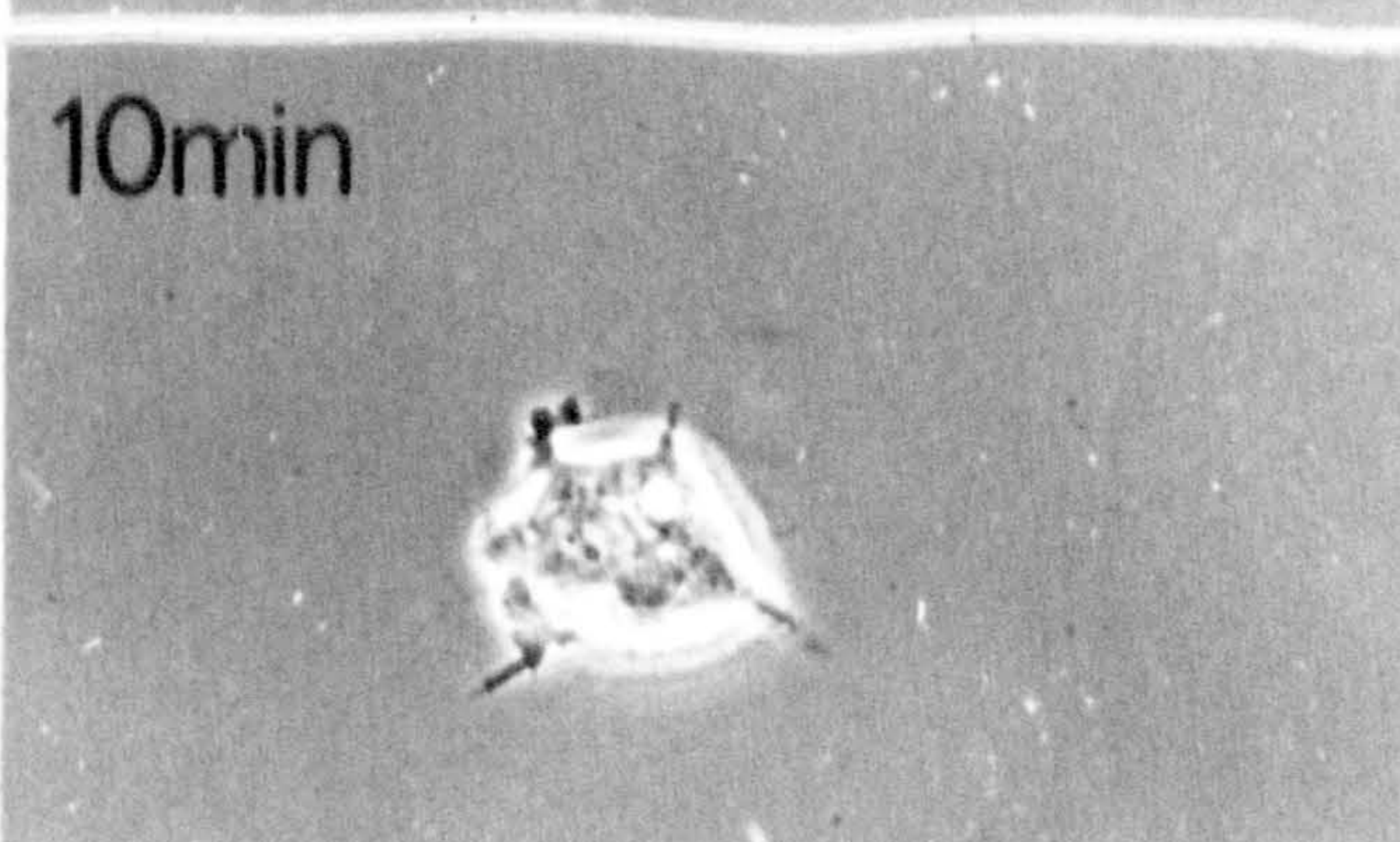
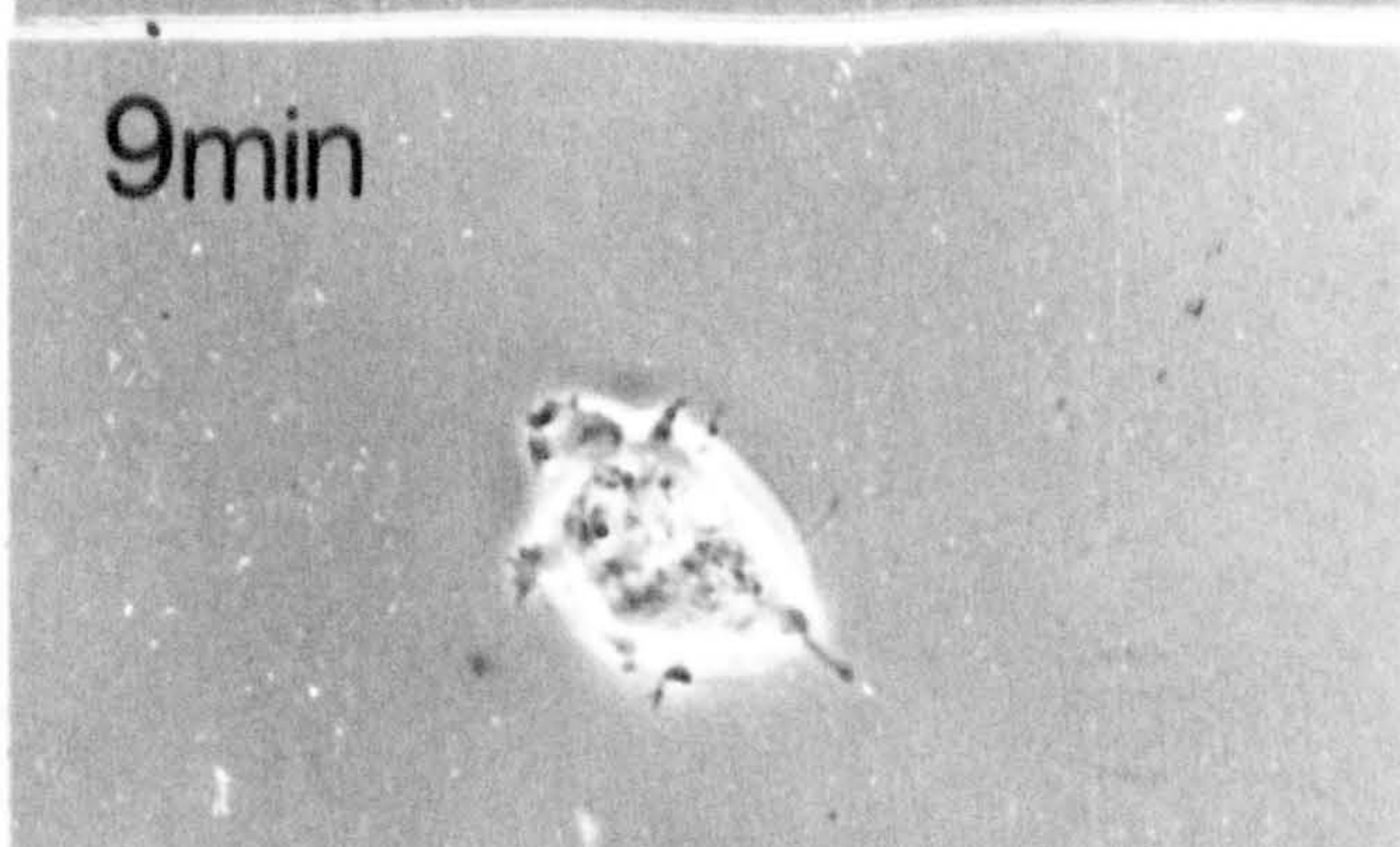
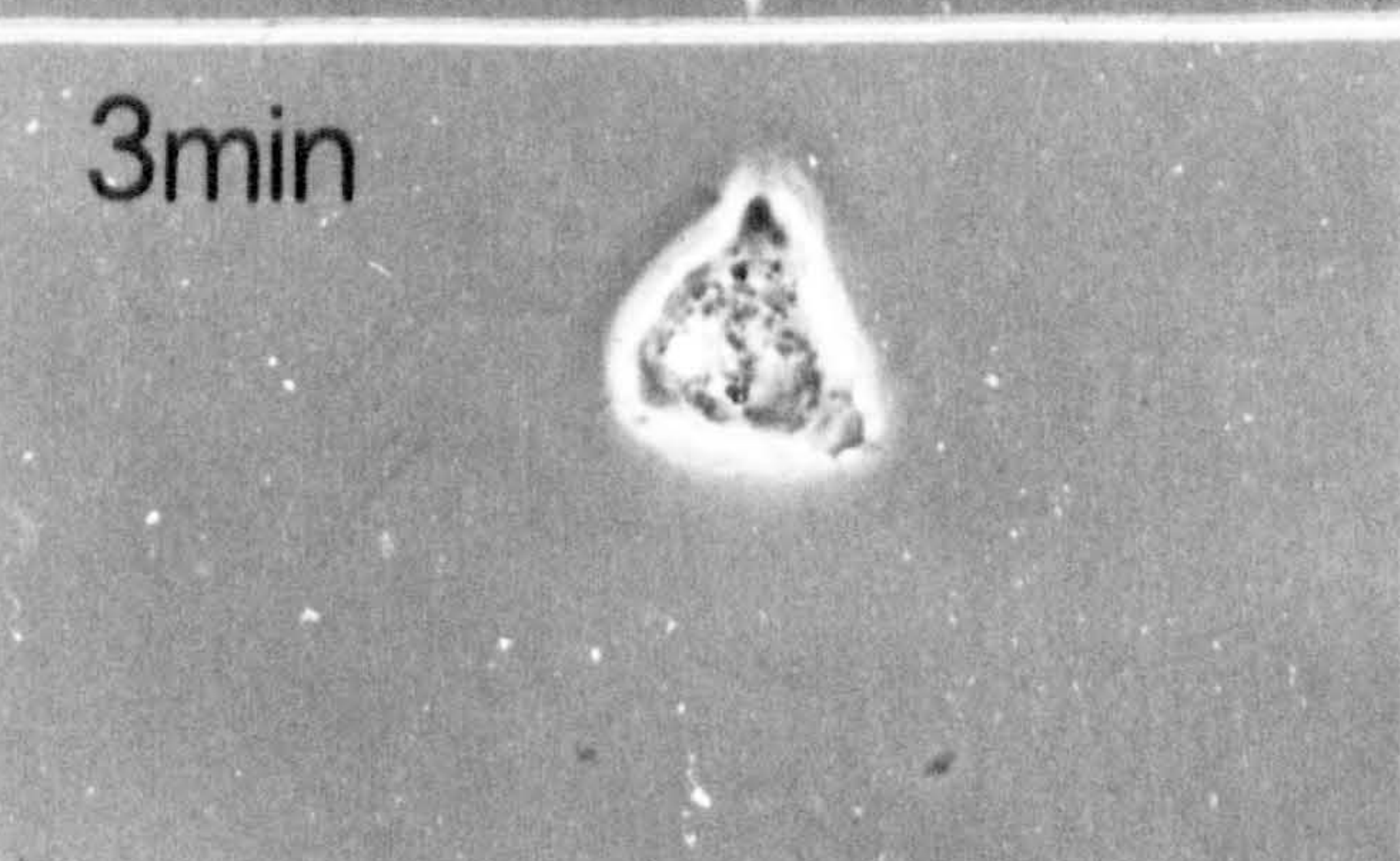
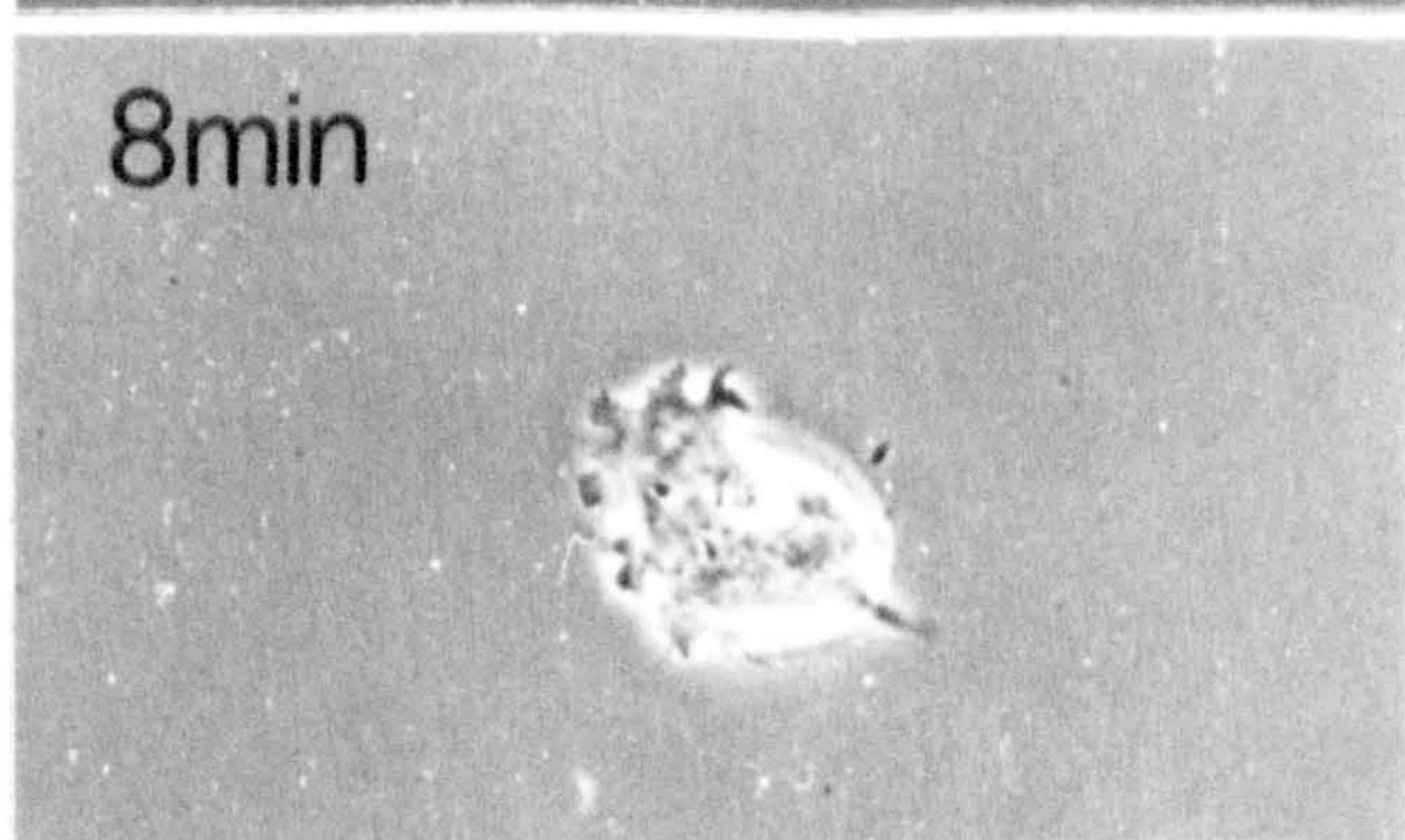
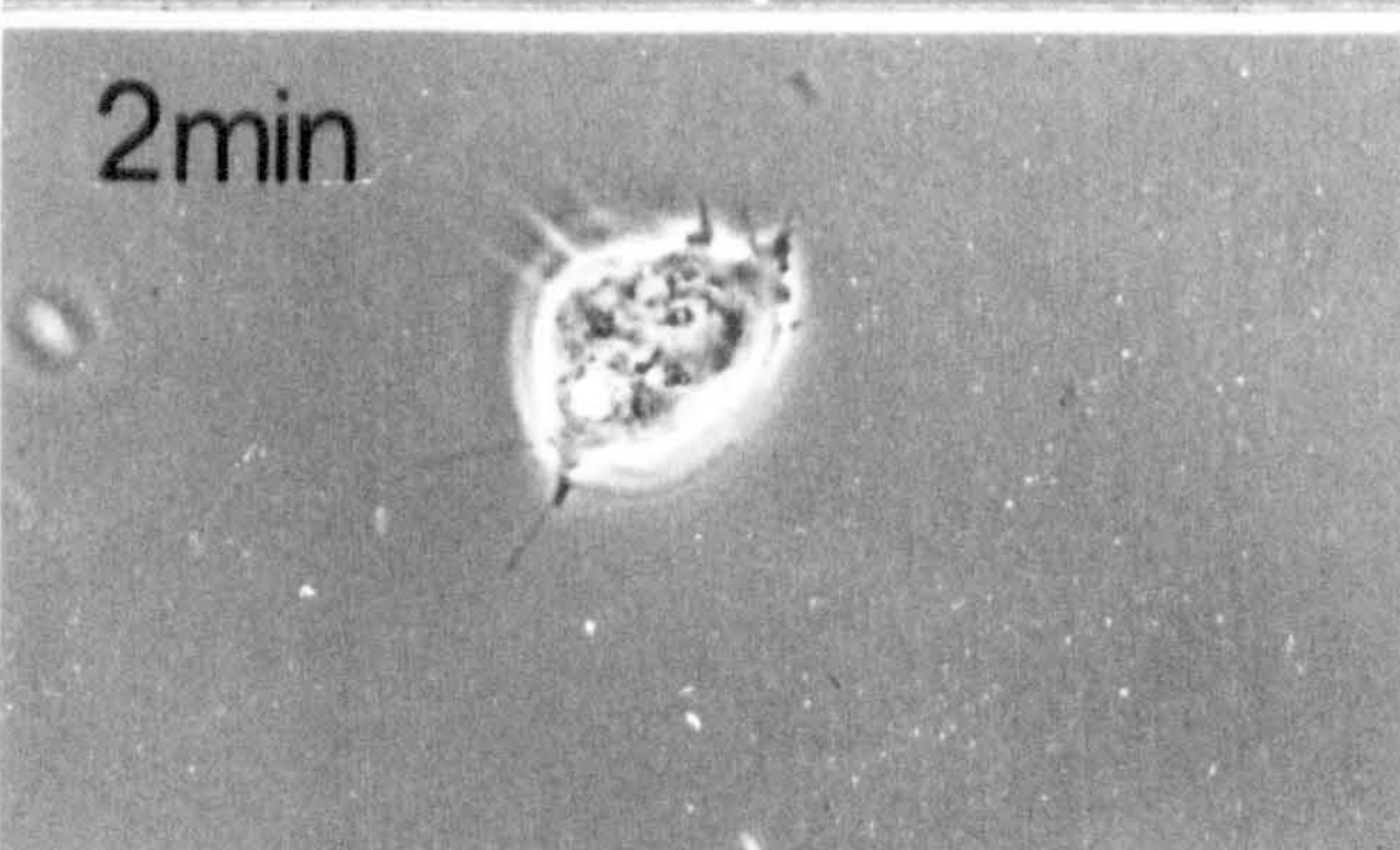
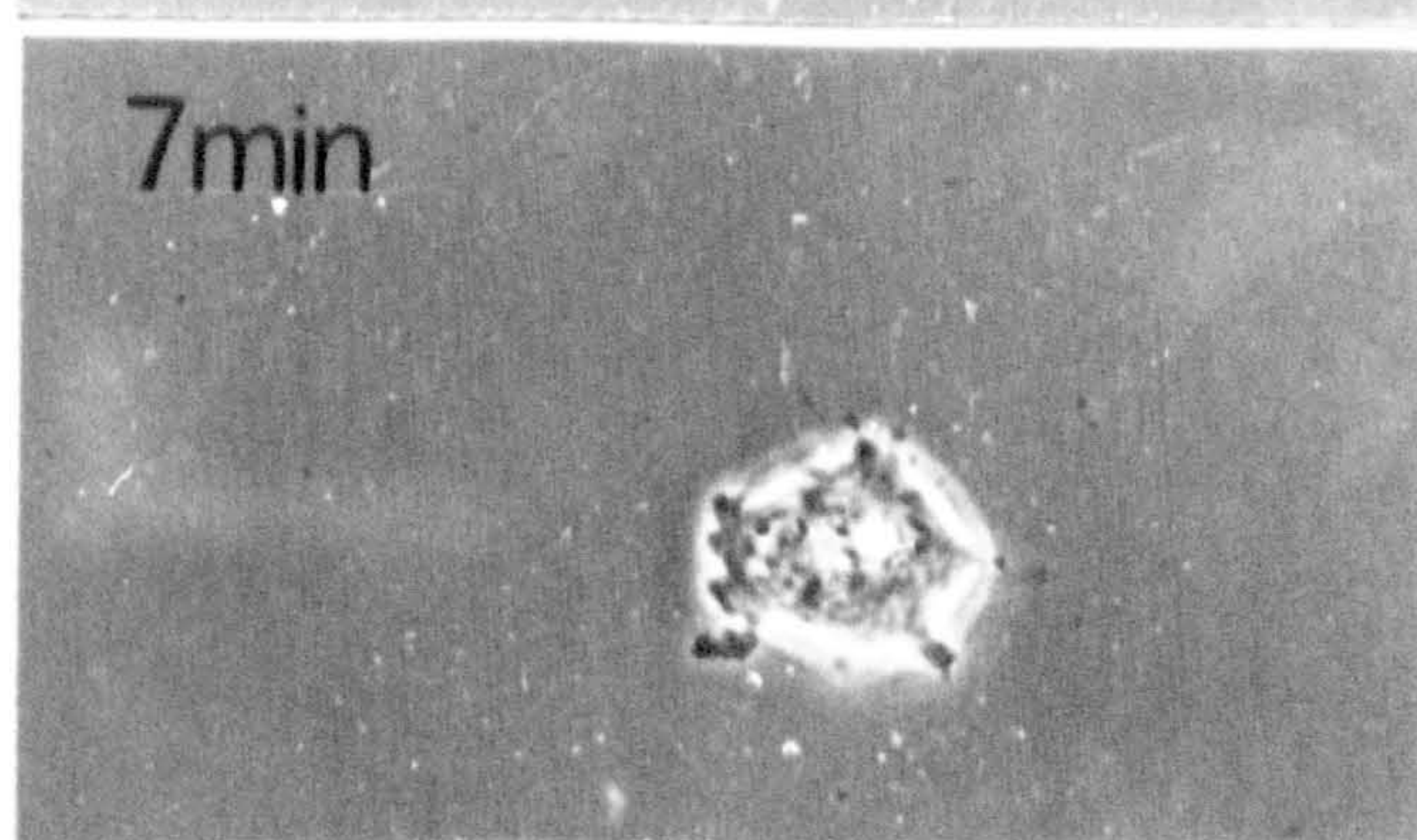
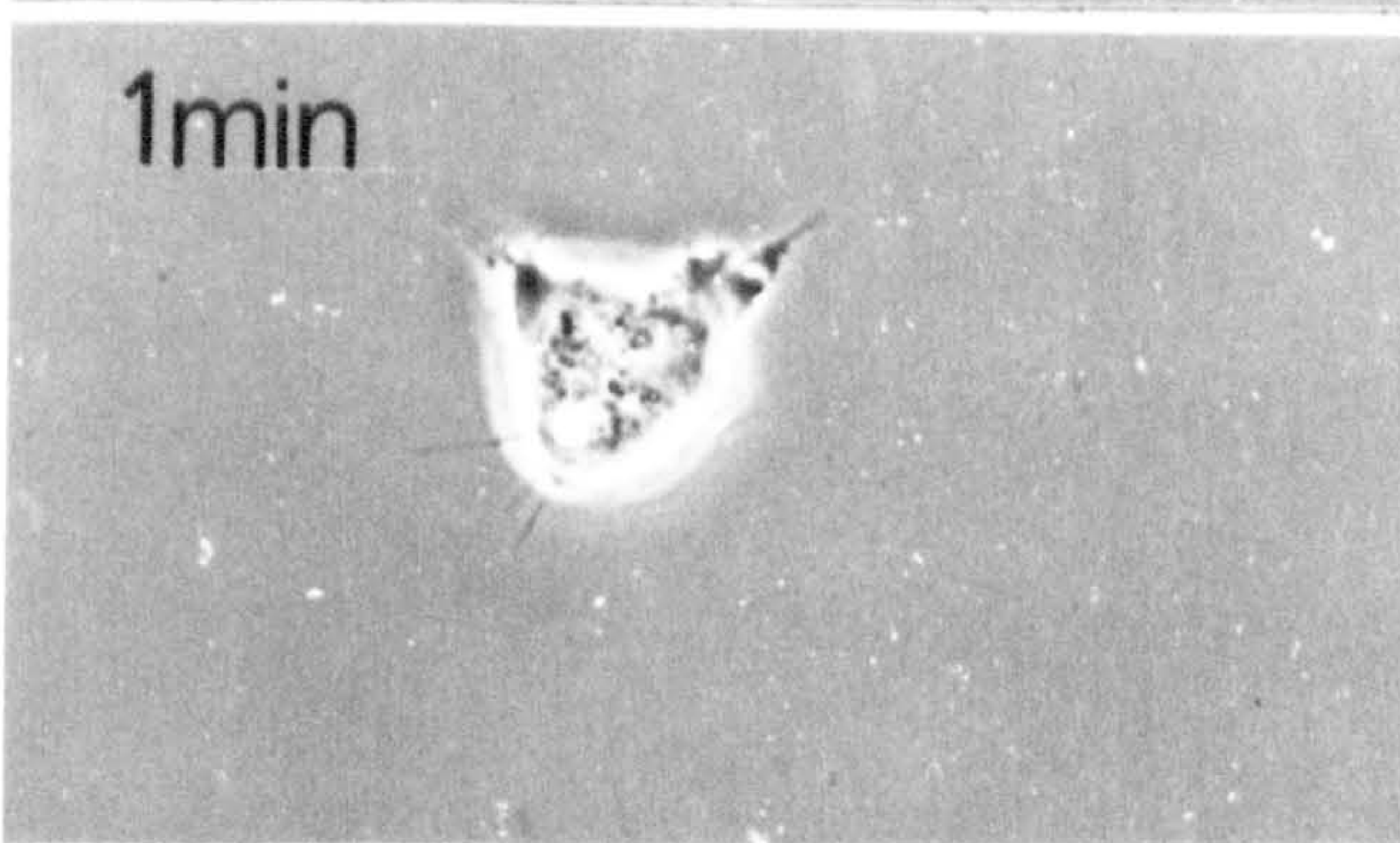
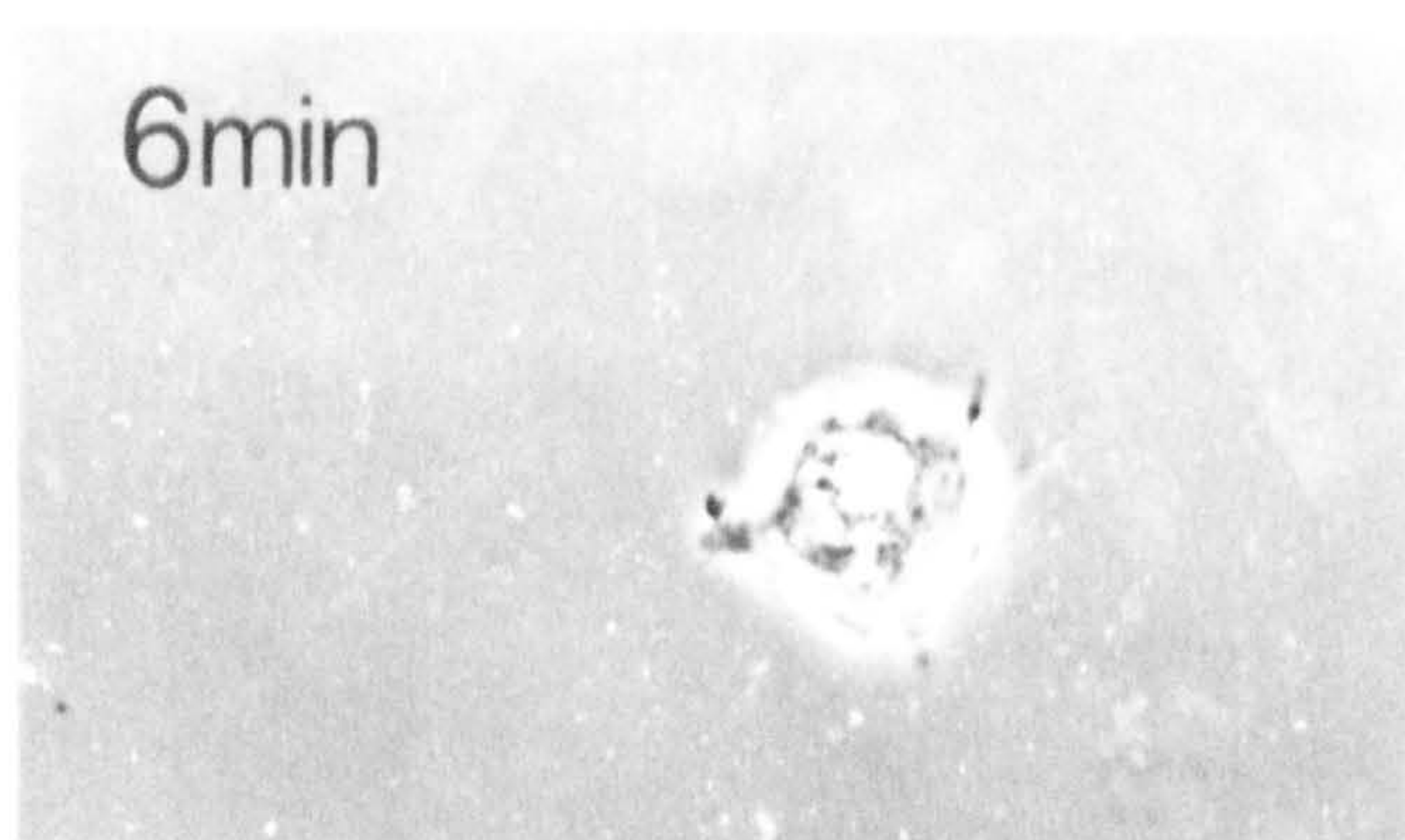
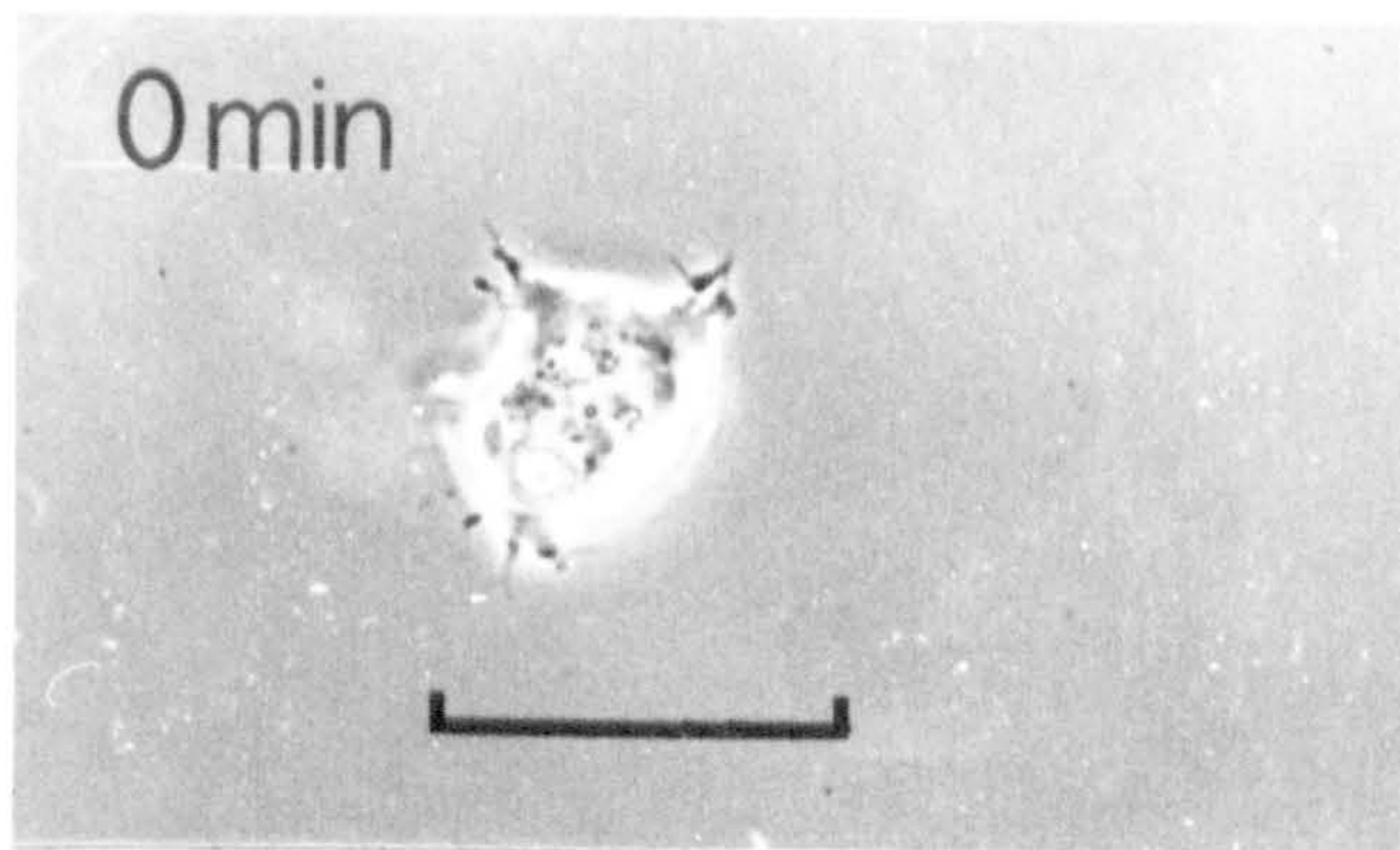
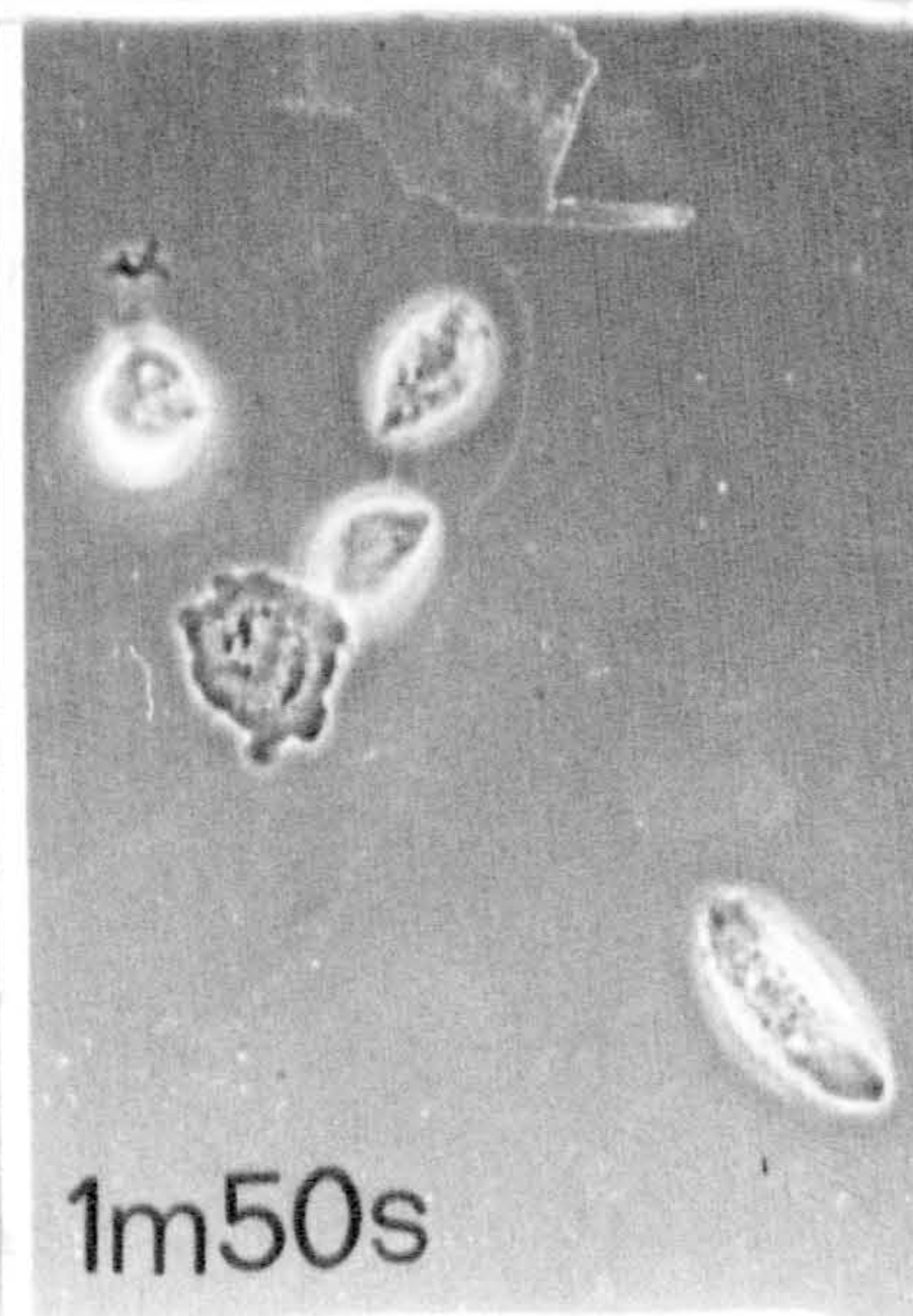
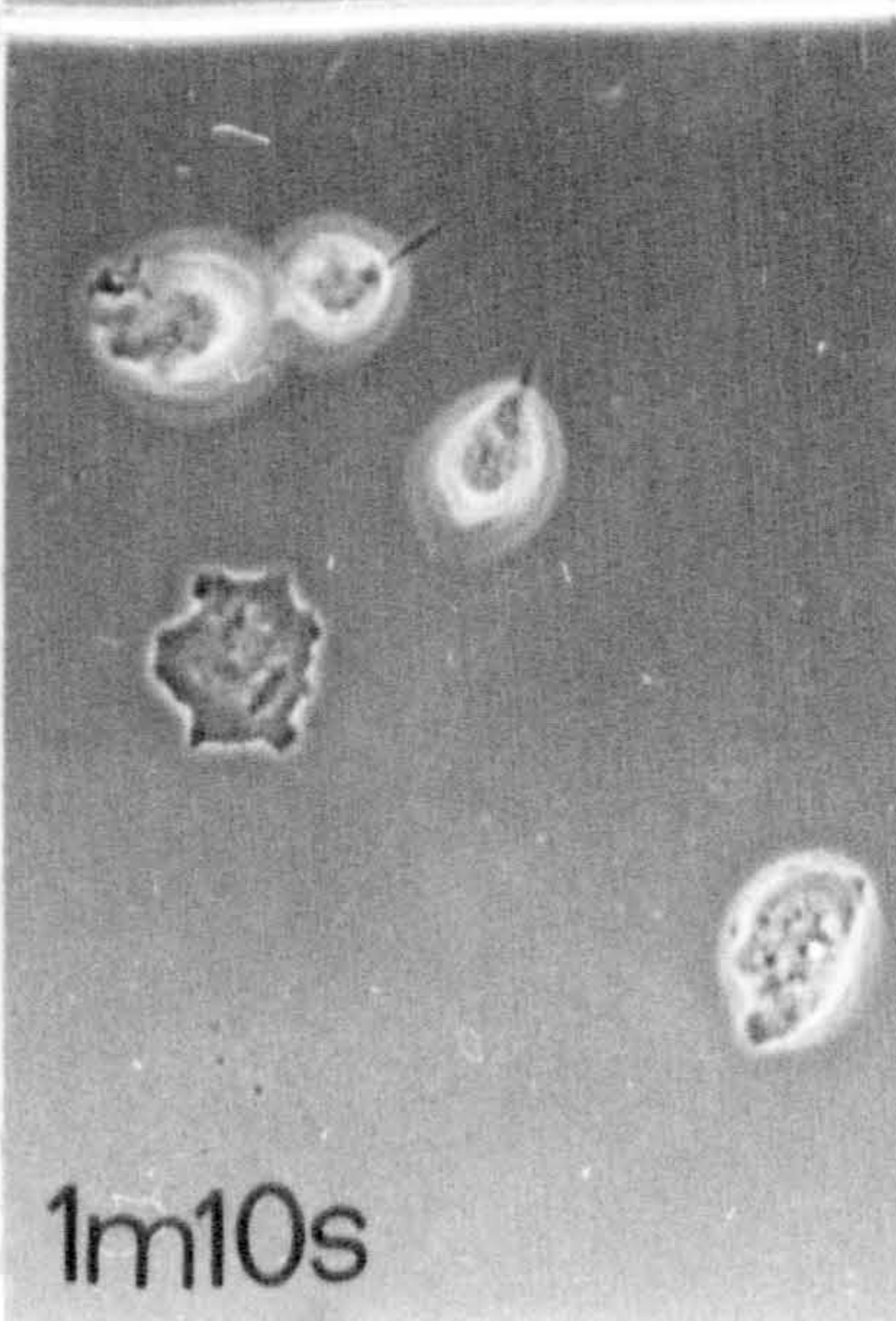
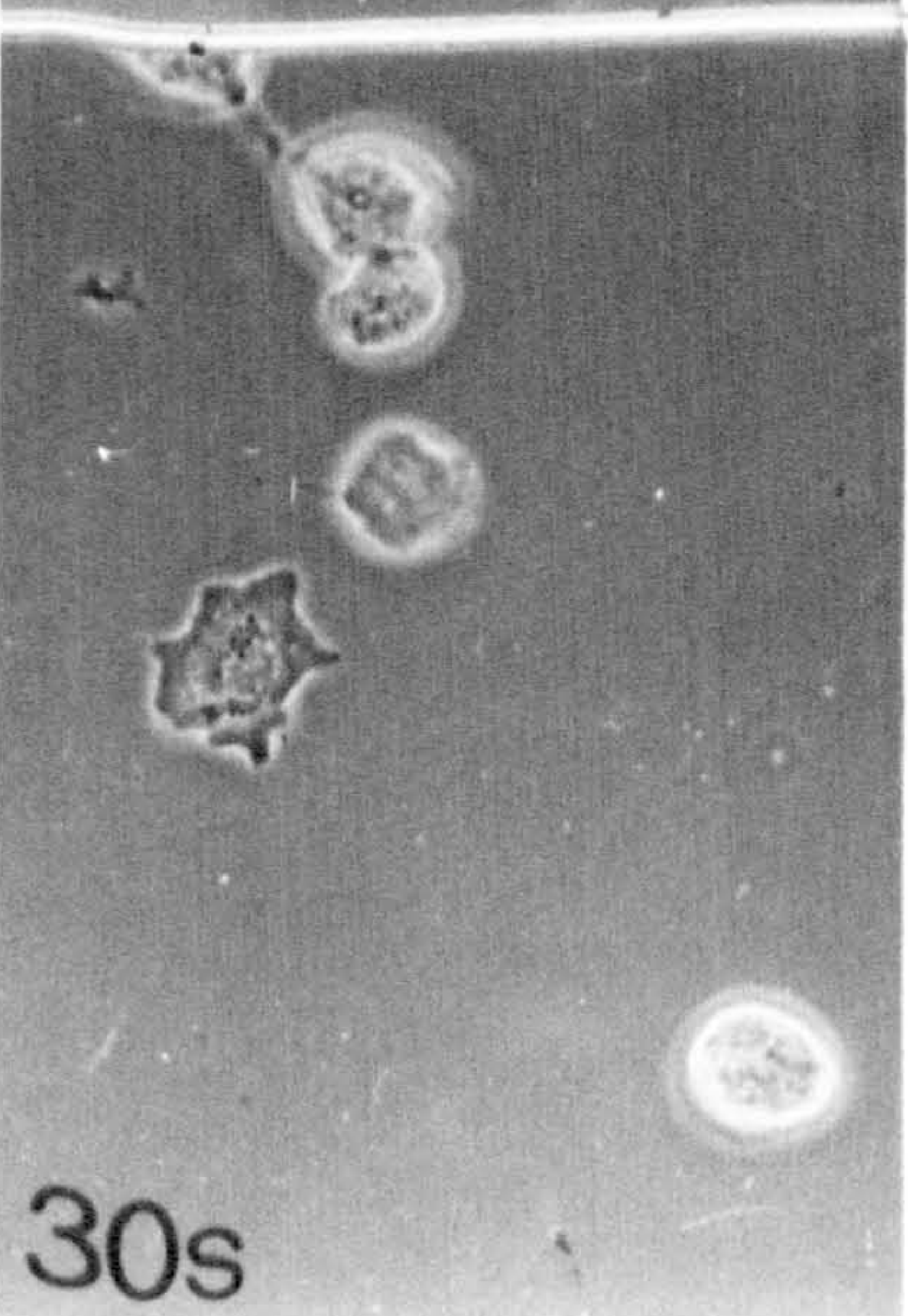
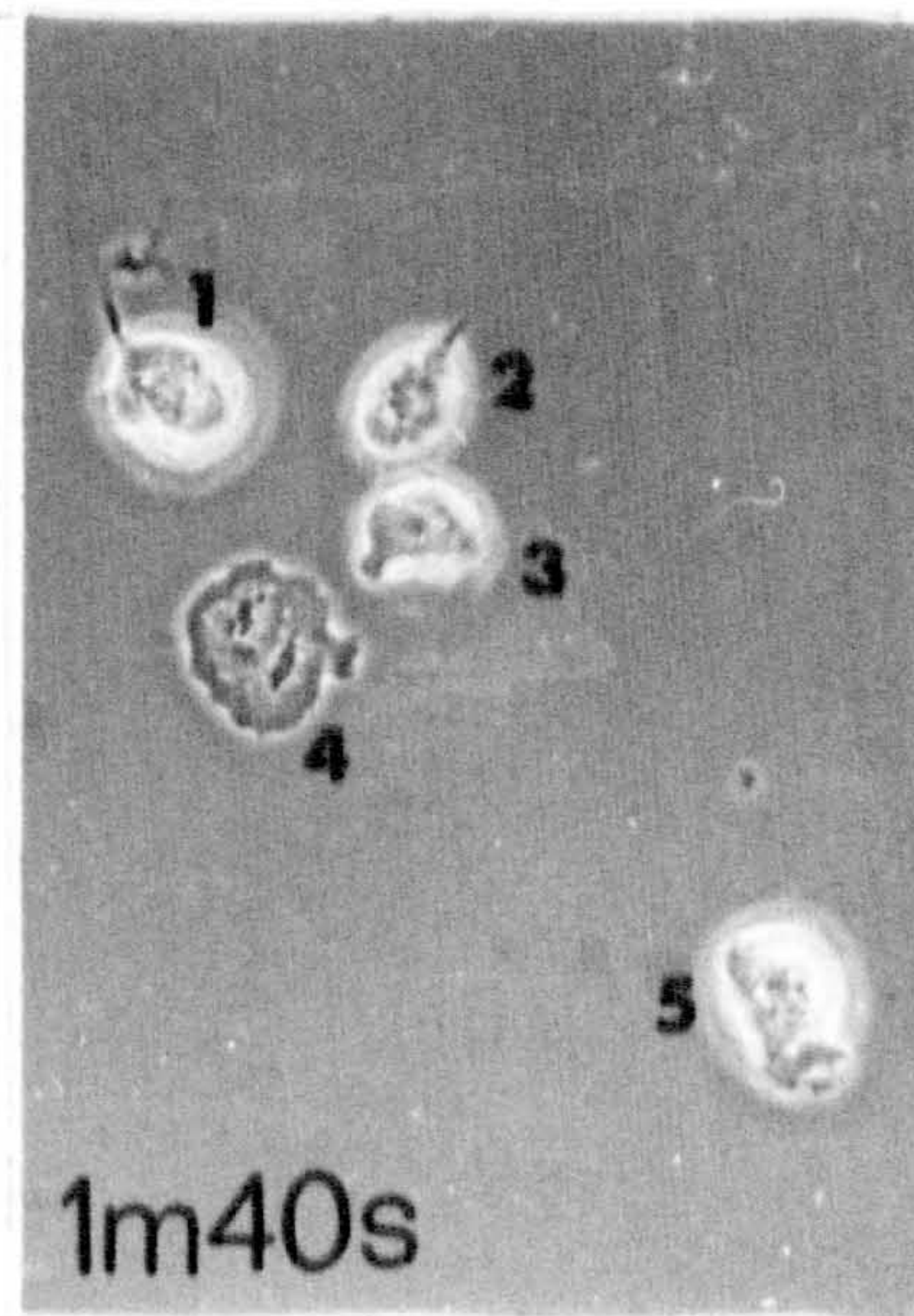
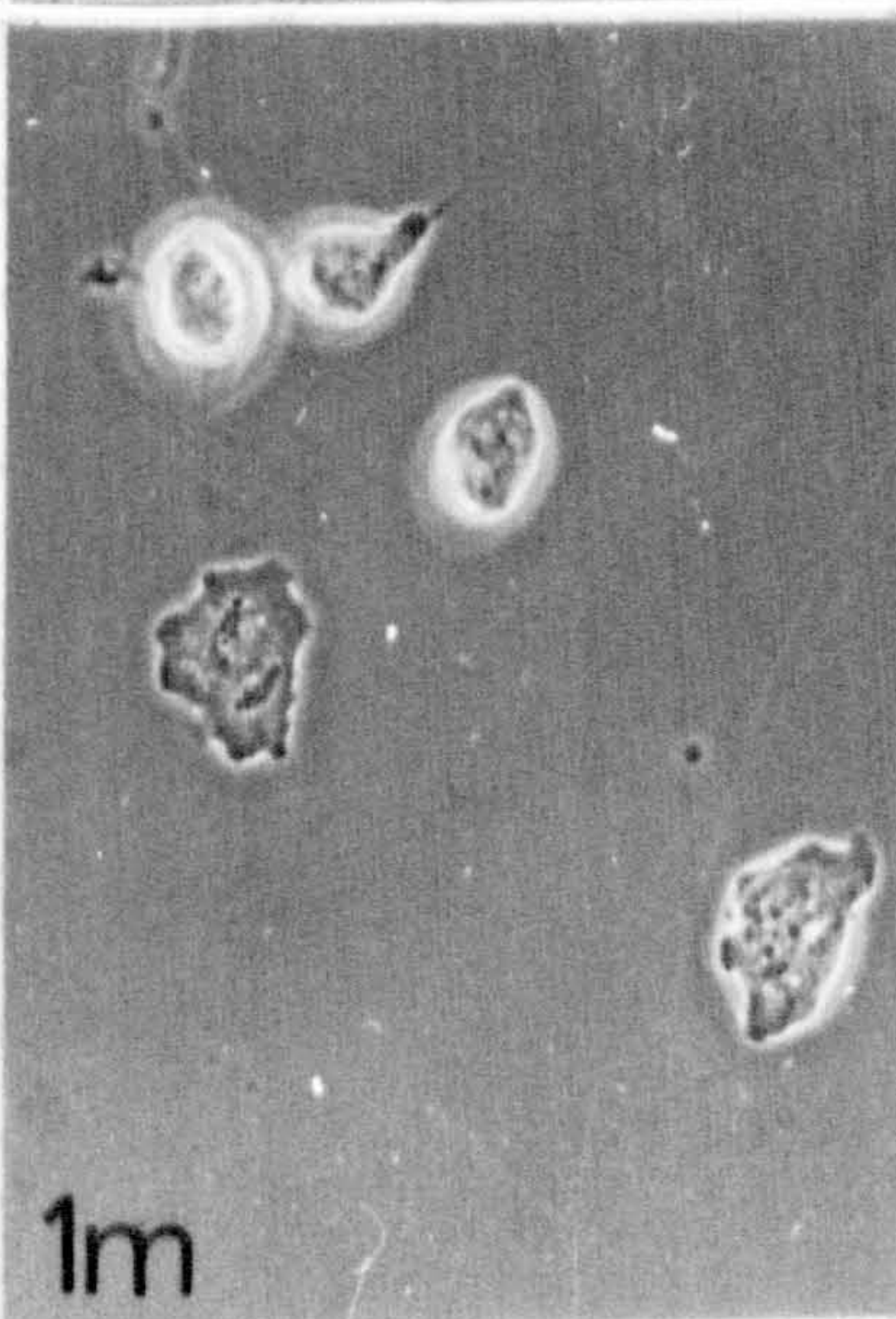
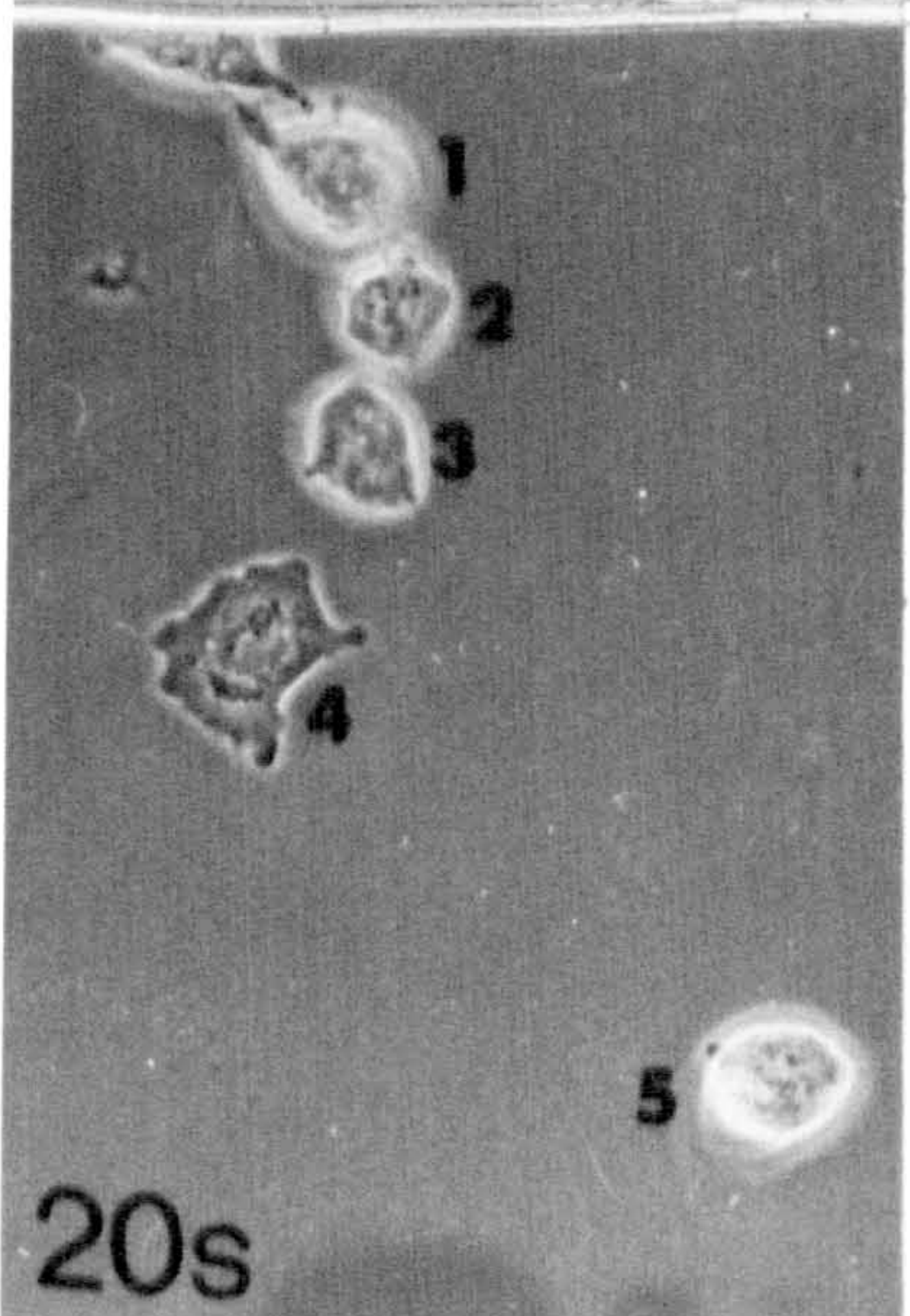
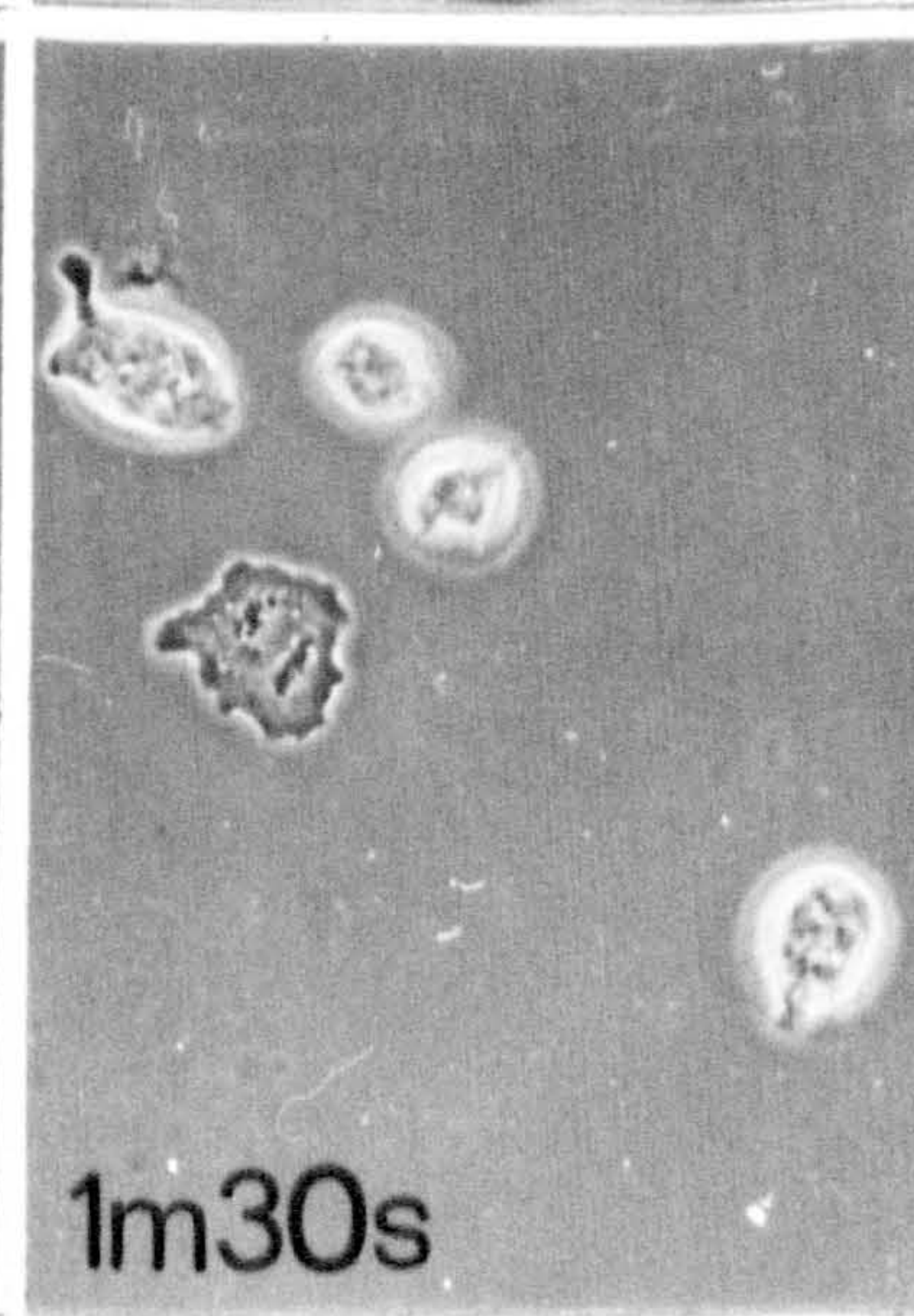
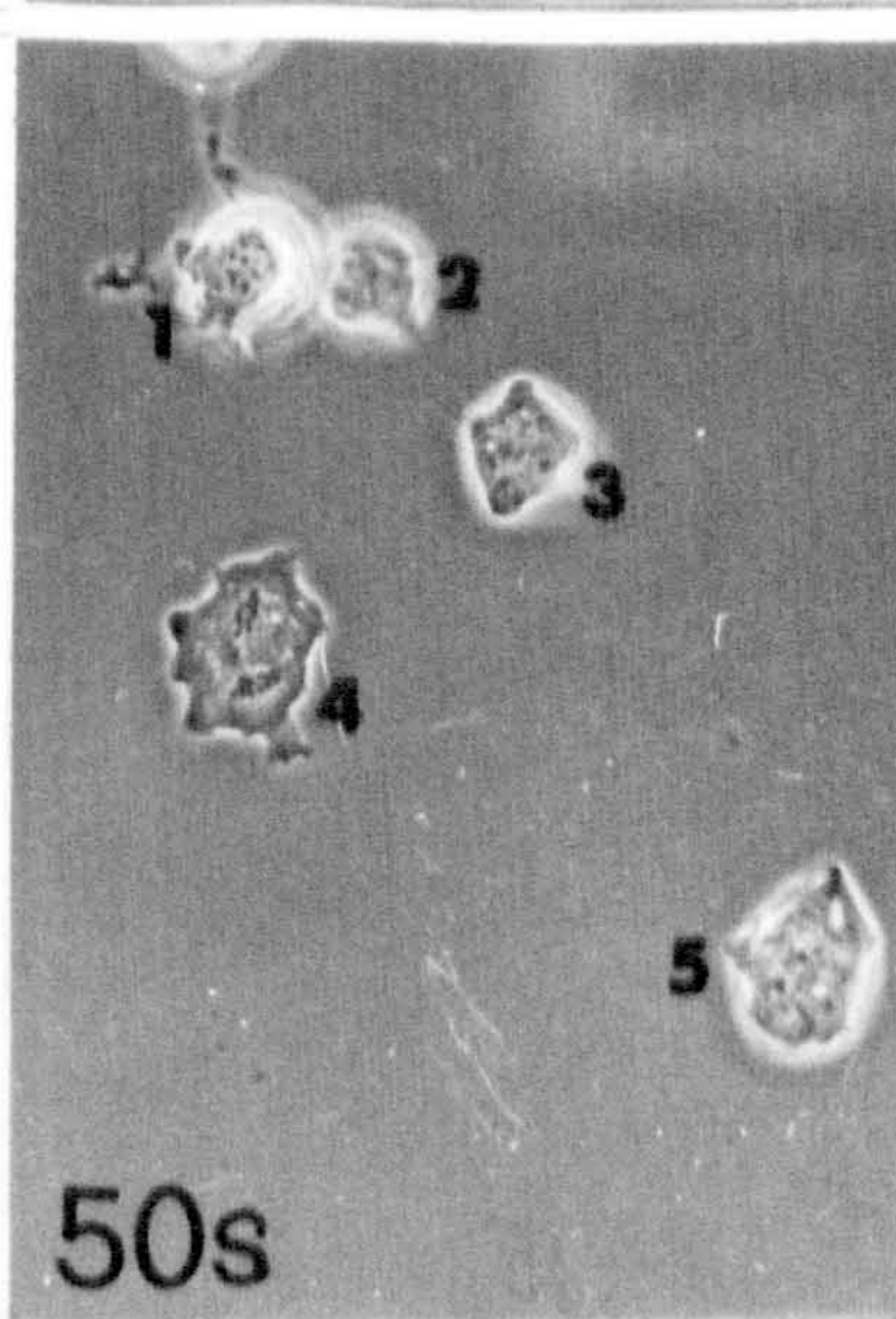
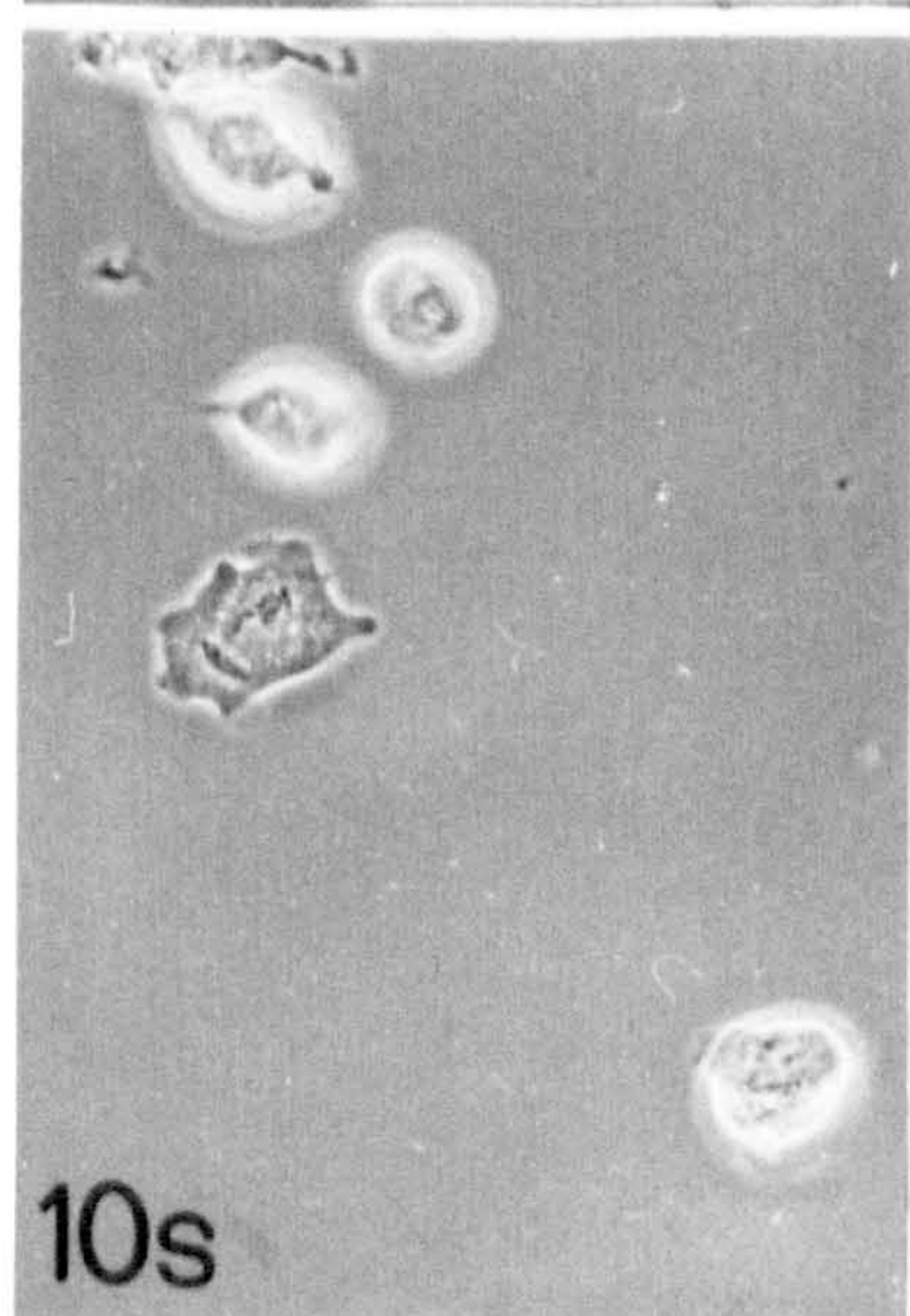
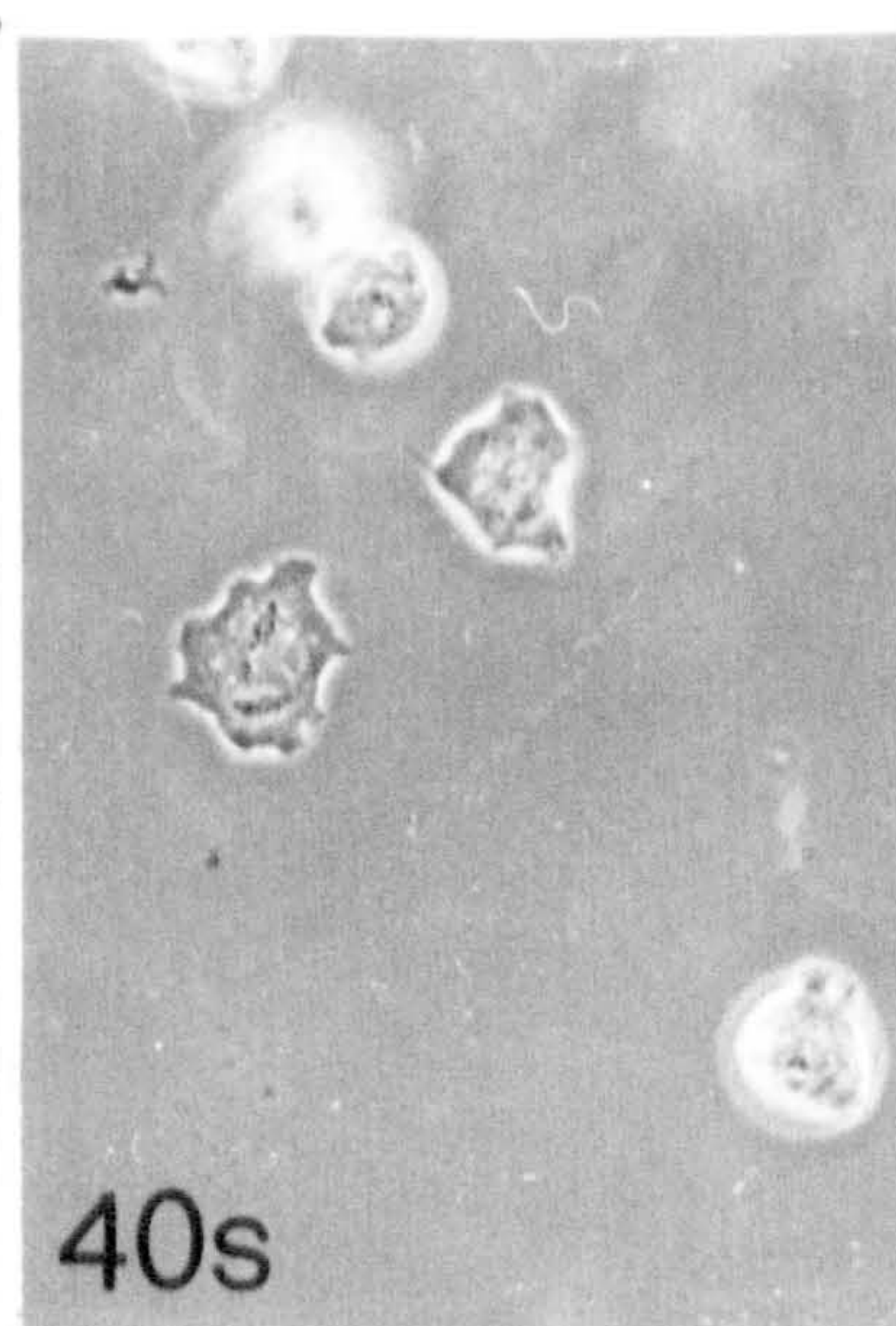
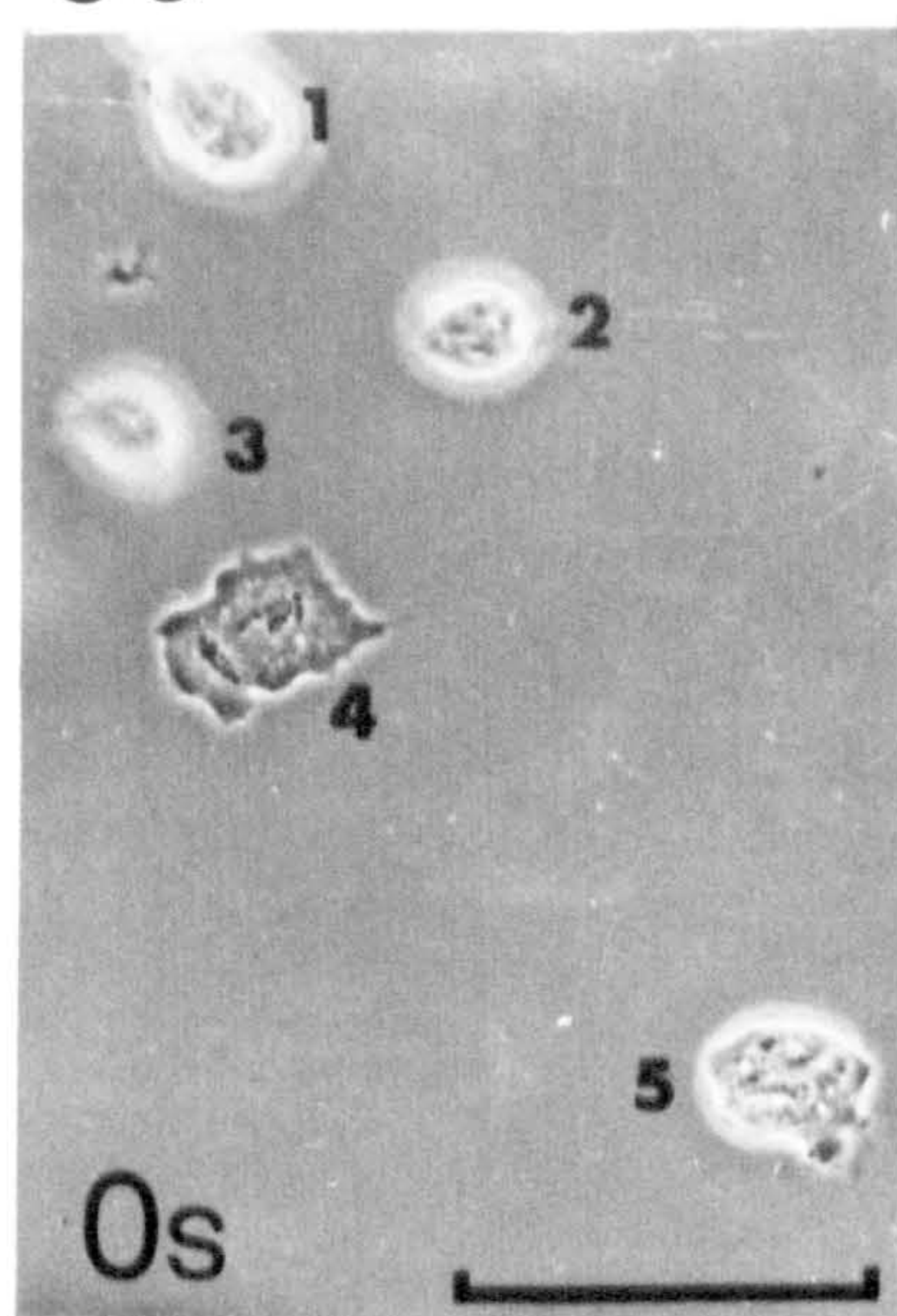




Fig. 96. A time-lapse sequence at 10 second intervals of phase contrast photographs of a group of living T. vaginalis which are adhering to a glass substratum in culture. The organisms have been numbered in some of the frames to enable the reader to follow the movements of individual organisms. This figure illustrates both the motility of T. vaginalis on a substratum and also the rapid changes in shape that may occur as the organisms move. For example the trichomonad numbered 5 (T5) changes from a flattened form to a spherical form between frames 0 seconds to 30 seconds but in the following 30 second period, to frame 1 min., it regains its flattened form. T4 remains flattened throughout the sequence. T1, T2 and T3 also change their degree of flattening as they move over the substratum; these three trichomonads move at speeds of up to 25  $\mu\text{m}$  per min.

Bar = 50  $\mu\text{m}$







Figs. 97 to 100. Interference reflection photographs of amoeboid T. vaginalis moving on a glass substratum in culture, showing the separation of the organisms from the substratum. See text for explanation of the technique. Each figure shows the phase contrast (PC) and interference reflection (IR) images of the trichomonads.

Bar = 20  $\mu$ m

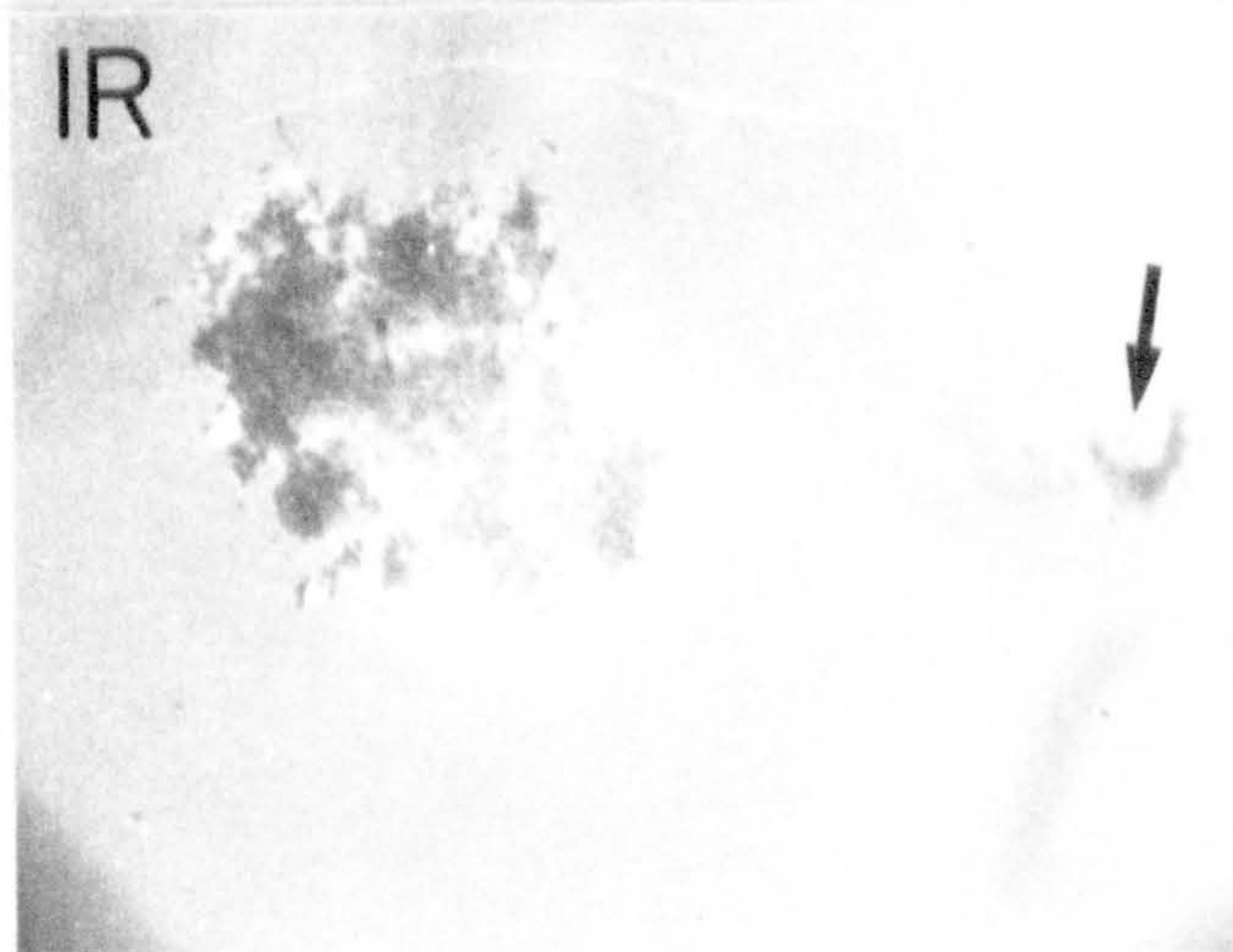
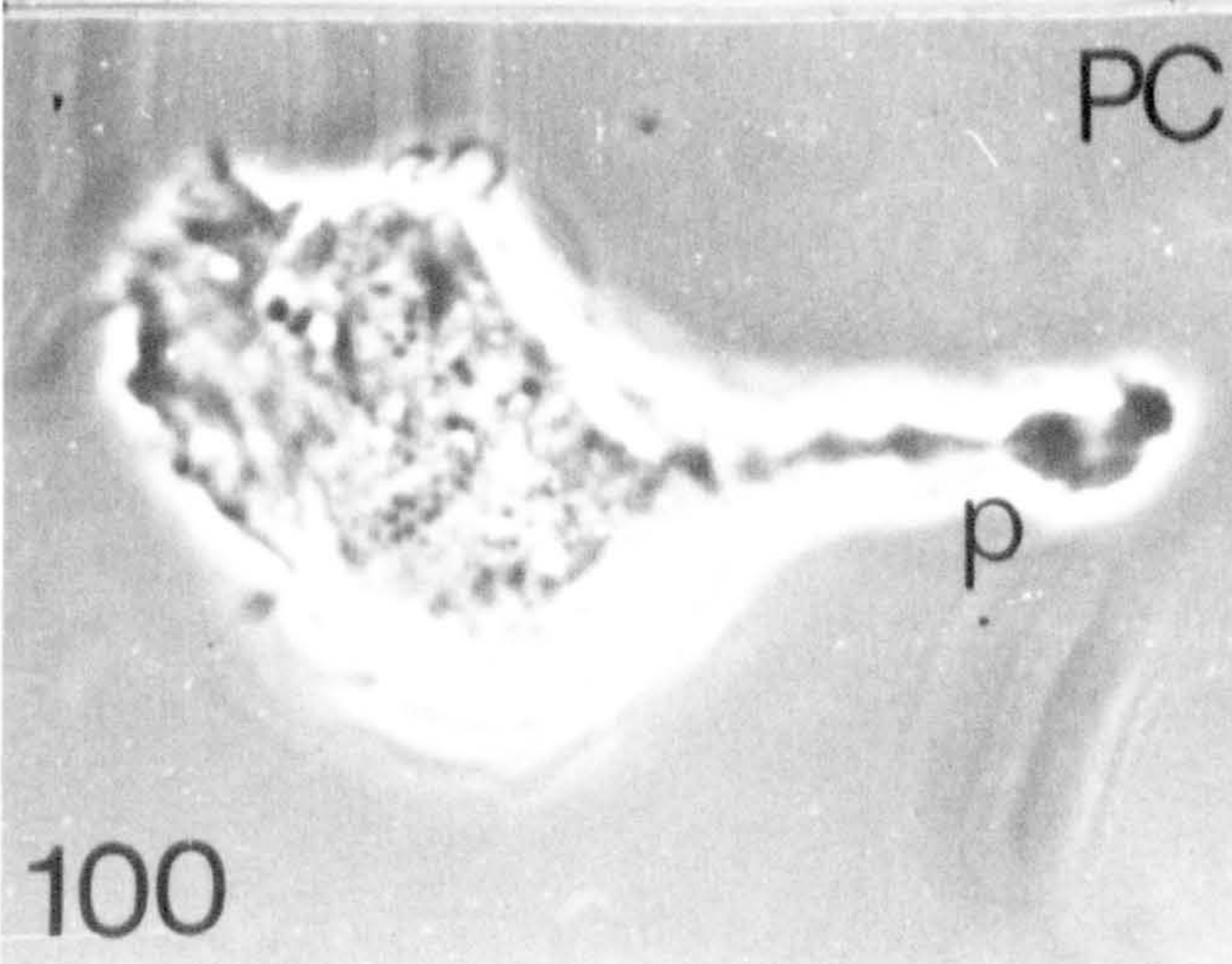
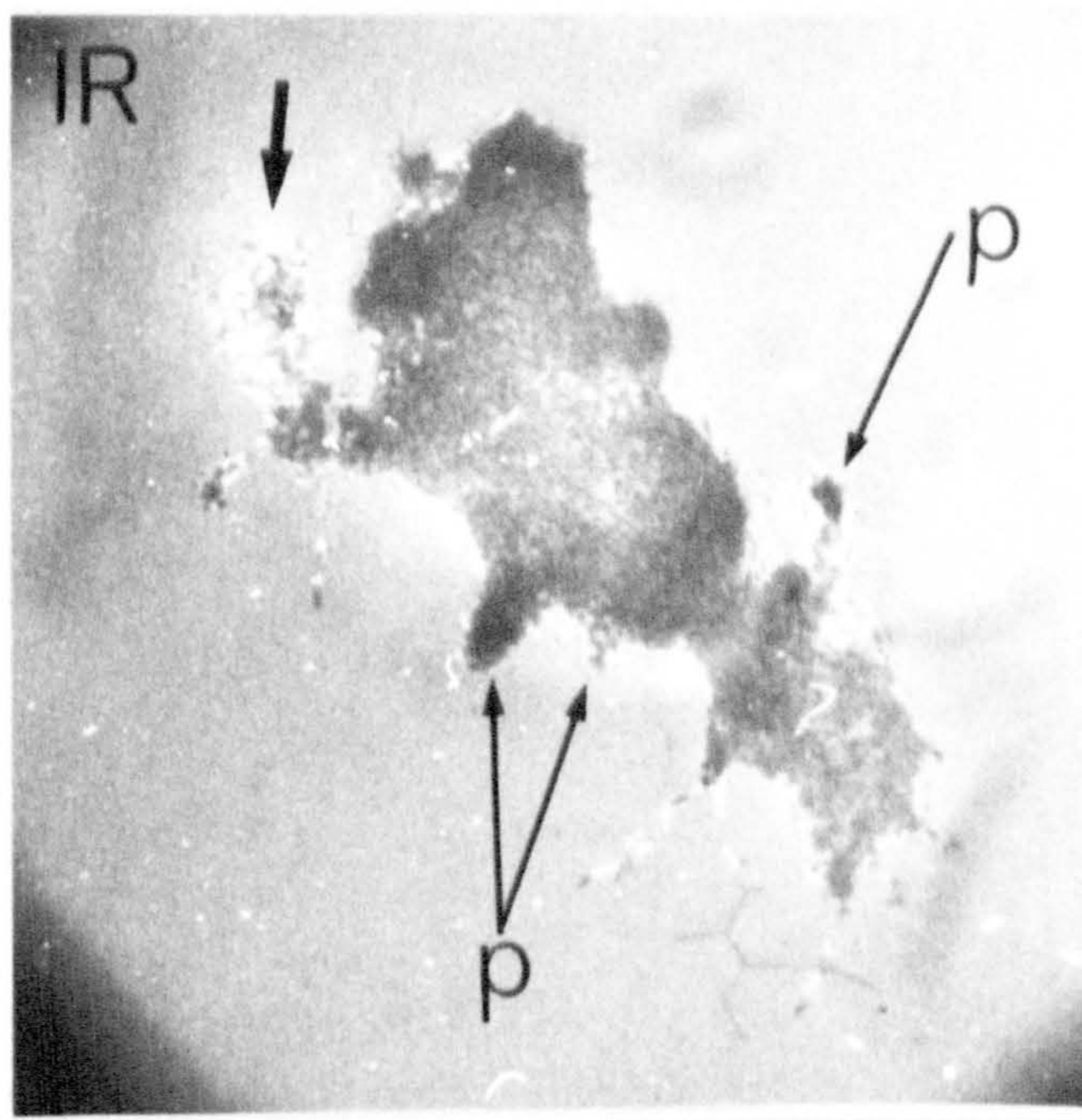
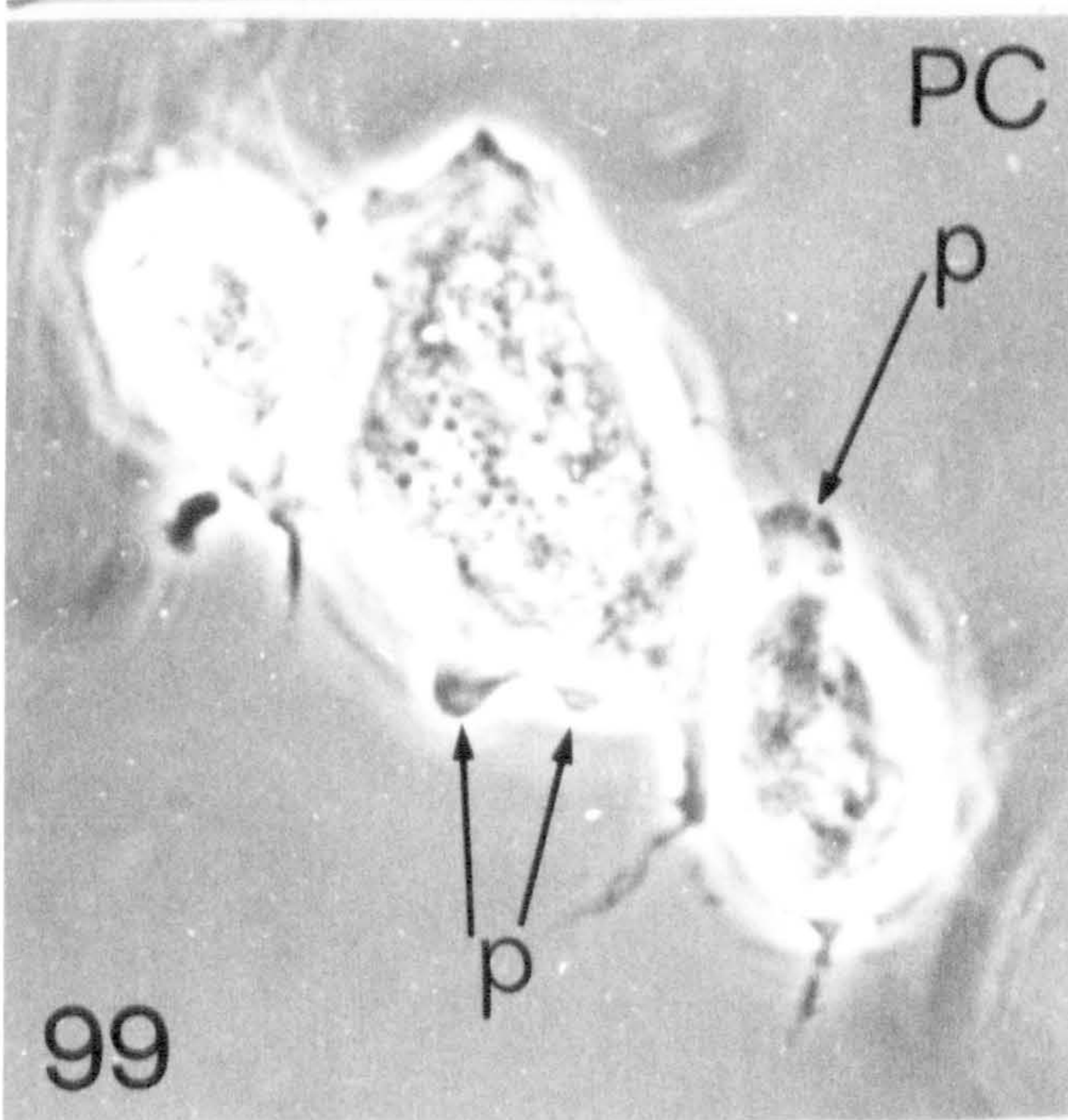
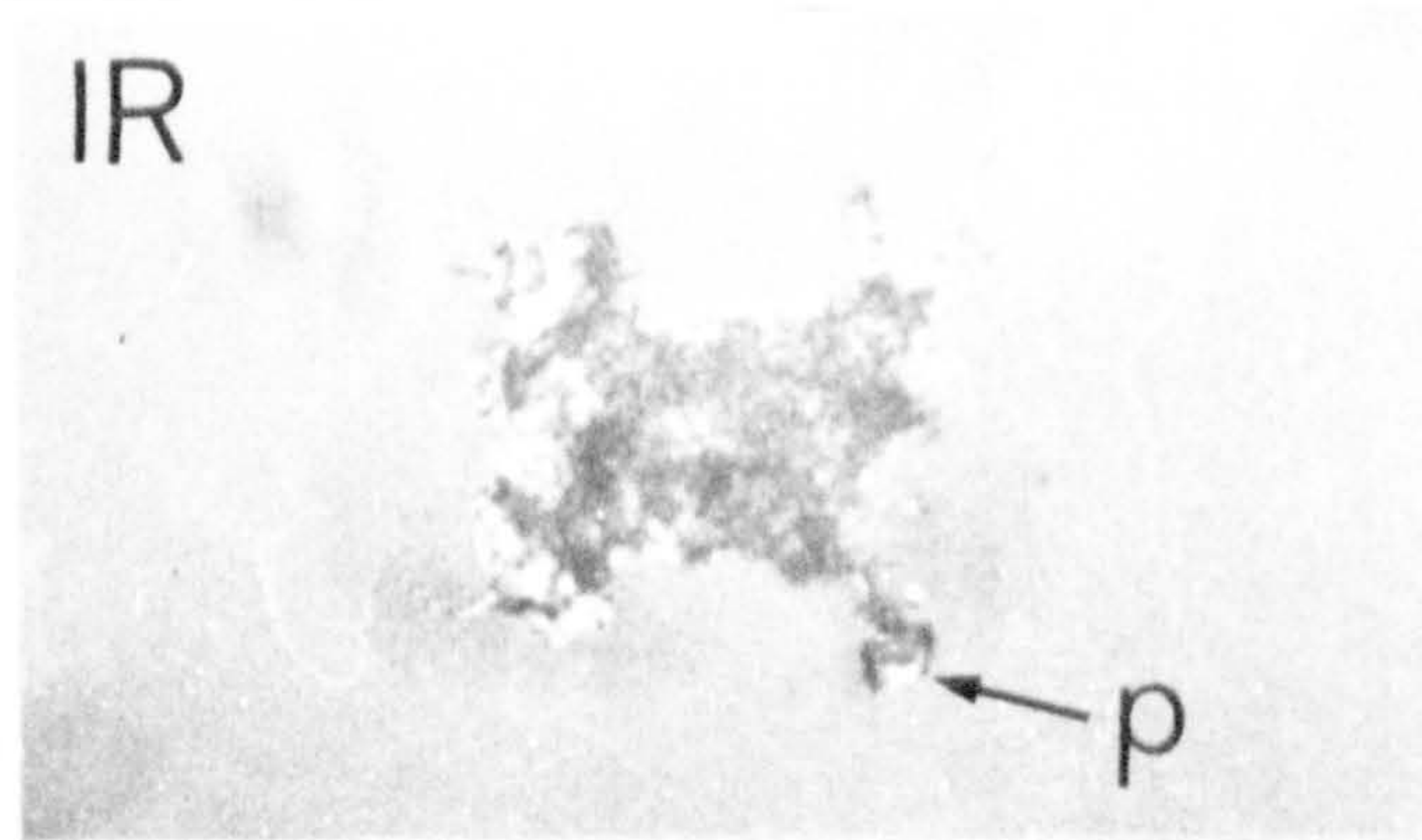
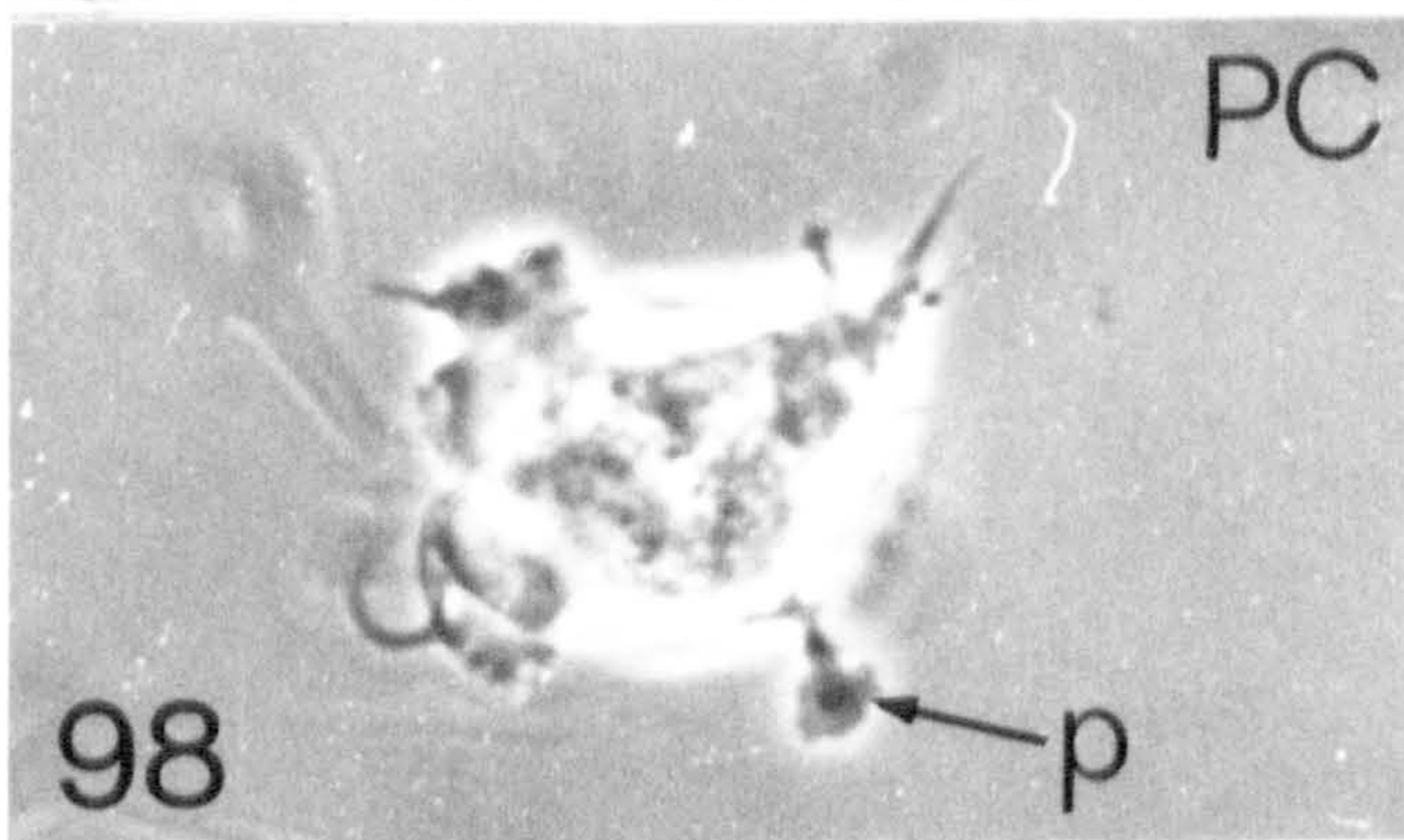
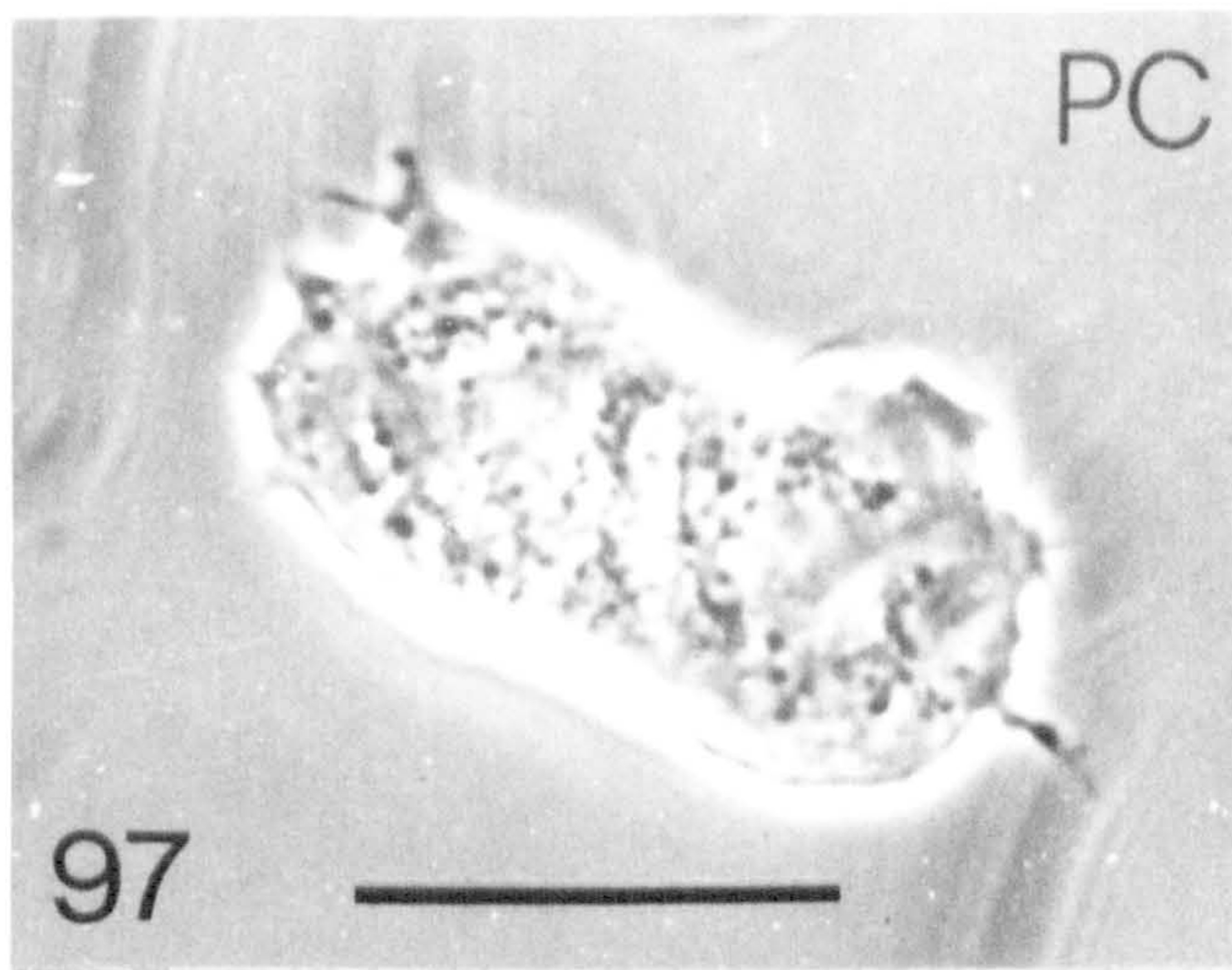
Fig. 97. A flattened amoeboid T. vaginalis. In IR the lower surface gives a uniform dark grey image indicating a separation of 10 to 30 nm. Note that the areas of the PC and IR images are similar showing that most of the lower surface of the organism is in contact with the substratum.

Fig. 98. A rounded T. vaginalis with three small pseudopodia (p). The IR image shows that although much of the body and pseudopodia lie within 30 nm of the substratum (grey areas) there are several areas which are about 100 nm from the glass (white areas).

Fig. 99. A group of three T. vaginalis. The central and righthand organisms have protruded pseudopodia (p) which the IR image shows to be lying within 10 to 30 nm of the substratum. However the organism on the right is barely visible in IR (arrow) although it is clearly seen in PC by its circular phase-contrast halo. This shows that this organism can have only a relatively small part of its surface in contact with the substratum.

Fig. 100. T. vaginalis with a long posterior pseudopodium (p). Note that only the distal tip of the pseudopodium is in contact with the substratum (arrow); the central part of the pseudopodium does not appear in the IR image showing that it must be lying 150 nm or more from the substratum. The body of this organism gives a grey to white image in IR indicating that the lower surface is not uniformly in contact with the glass.







## Ultrastructure of *T. vaginalis*

TO THE EDITOR *British Journal of Venereal Diseases*

SIR—The paper by Ovčinnikov, Delektorskij, and Kosmacheva (1974) requires some comment in the light of recent advances in the study of the ultrastructure and biochemistry of *Trichomonas vaginalis* and other trichomonad species, including the urogenital parasite of cattle *Trichomonas foetus*.

My work in this laboratory on the ultrastructure of *T. vaginalis* confirms that reported by Nielsen, Ludvik, and Nielsen (1966) with regard to the association of the flagellar apparatus and the axostyle. The axostyle is a single sheet of microtubules running parallel to the longitudinal axis of the cell. The sheet is coiled like a cone at the posterior end where it projects from the cell for a distance of several microns; at the anterior end of the cell the sheet flattens out and terminates at the side of the kinetosomes of the five flagella. Lying next to, but apparently not attached to, the anterior end of the axostyle is a smaller sheet of microtubules running at right-angles to the axostyle and curving around the kinetosomes; this is known as the pelta. The organelle identified by Ovčinnikov and others (1974) as the 'parabasal apparatus'

is, I believe, the anterior end of the axostyle and the pelta and not, as they suggest, a separate structure.

The identification as lysosomes of the numerous electron dense granules with their single membrane and granular matrix, a characteristic component of the cytoplasm of trichomonads, lacks corroboration by other workers. Lysosomes have the common property of containing acid hydrolases encompassed in a semi-permeable limiting membrane. Their role in the cell is thought to involve the intracellular digestion of both endogenous and exogenous material (Cohn and Fedorko, 1969). Müller (1973) investigated the enzyme content of the different subcellular components of *T. foetus* and found that the electron dense granules contained no acid phosphatase, an enzyme marker of lysosomes, but that the enzyme was found in a larger population of granules more heterogeneous in size with pleomorphic contents, probably phagocytosed material. Subsequently, Lindmark and Müller (1973) named the electron dense granules of *T. foetus* as hydrogenosomes due to their involvement in the anaerobic trichomonads in the production of hydrogen. Brugerolle and Metenier (1973)

were able to demonstrate malate dehydrogenase activity in the electron dense granules of *T. vaginalis* and Brugerolle (1972) excludes the possibility that they are lysosomes. Müller (1973) demonstrated malate dehydrogenase and  $\alpha$ -glycerophosphate dehydrogenase in the electron dense granules of *T. foetus*. I have not seen in any of my electron micrographs of *T. vaginalis* any evidence of fusion of the electron dense granules with vacuoles containing phagocytosed material, a process primary lysosomes undergo in the formation of secondary lysosomes (De Duve and Wattiaux, 1966).

Thus the evidence suggests that the electron dense granules of trichomonads are involved in the energy producing mechanisms of the cell and they should not be labelled as lysosomes. It is likely that the numerous small vesicles, and the Golgi apparatus from which they are thought to have originated, participate in the digestion of phagocytosed material in *T. vaginalis*.

Further research is needed on the cytoplasmic contents of *Trichomonas vaginalis* to elucidate their role in the

metabolism of the parasite and in the pathogenicity of trichomoniasis.

Yours faithfully,

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